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Distal Recognition Site for Classical Pathway Convertase Located in the C345C/Netrin Module of Complement Component C5

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Previous studies focused on indels in the complement C345 protein family identified a number of potential protein-protein interaction sites in components C3 and C5. Here, one of these sites in C5, near the α-chain C terminus, was examined by alanine-scanning mutagenesis at 16 of the 18 non-alanine residues in the sequence KEALQIKYNFSFRYIYPLD. Alanine substitutions affected activities in the highly variable manner characteristic of binding sites. Substitutions at the lysine or either phenylalanine residue in the central KYNFSF sequence had the greatest effects, yielding mutants with <20% of the normal activity. These three mutants were also resistant to the classical pathway (CP) C5 convertase, with sensitivities roughly proportional to their hemolytic activities, but had normal susceptibilities to the cobra venom factor (CVF)-dependent convertase. Synthetic peptide MGKEALQIKYNFS-NH₂ was found similarly to inhibit CP but not CVF convertase activation, and the effects of alanine substitutions in this peptide largely reflected those of the equivalent mutations in C5. These results indicate that residues KYNFSF form a novel, distal binding site for the CP, but not CVF convertase. This site lies ~880 residues downstream of the convertase cleavage site within a module that has been independently named C345C and NTR; this module is found in diverse proteins including netrins and tissue inhibitors of metalloproteinas.


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The molecular basis for specificity of the activating proteases is unknown. However, in previous work, we found that the susceptibility of C4 to C1s activation can be almost completely eliminated by deletion of three residues near the activation site (3) and, conversely, that C3 and C5 can both be rendered susceptible to C1s by substituting C4-like sequences near their activation sites (4, 5). These results indicated that recognition by C1s primarily involves interactions with residues flanking the cleavage site. The latter studies also revealed unexpectedly that the same sequence changes that allowed cleavage of C3 and C5 by C1s did not substantially interfere with activation by their own convertases. Hence, these mutants had lost the ability to discriminate between C1s and their own convertases. This was particularly striking in the case of C5, where sensitivity to C1s was achieved only after insertion of 2 residues and replacement of 20 others flanking both sides of the cleavage site. Even with these extensive changes, the convertase sensitivity of the mutant was indistinguishable from that of wild-type (wt) C5. To explain these observations, we proposed that recognition of C3 and C5 by their specific convertases involves interactions distal to their cleavage sites (5).

The large sizes of C3 and C5 (>1600 residues) made an undirected search for these putative distal recognition sites impractical. Therefore, we tried to expedite this search by focusing on sites near indels in the protein family composed of C3, C4, and C5 (6). Indels are the evolutionary insertions or deletions of amino acid residues that result in length polymorphisms among members of a
protein family. They are usually found at the protein surface, and hence we reasoned that they should be good starting points in a search for intermolecular binding sites (7). To test this idea, we used mutational and peptide inhibition strategies to “scan” the larger (≥2 residues) indels in C3 and C5 for potential interaction sites (6, 8, 9). These studies identified a number of candidate sites and provided a foundation for broader studies, because they were designed to locate all sites important for complement lytic activity, not just those involved in proteolytic activity. They could not definitively establish which of these candidates were genuine protein-protein interaction sites because the peptide approach depended on the precarious assumption that synthetic peptides adopt the activities of the corresponding sequences in the intact protein, and the mutational strategy involved relatively disruptive insertion and deletion mutations that were designed to maximize the chances of finding a binding site, while minimizing the number of mutants to be constructed and analyzed (8). Therefore, it was quite possible that the peptide results were caused by activities unrelated to those of the corresponding sequence in the intact protein and that the mutational effects were caused by long range structural perturbations rather than to sequence changes at a genuine binding site.

In the present study, we used alanine-scanning mutagenesis (10) to test whether one of the candidate binding sites in C5, near the α-chain C terminus at indel 26 in our designation (6), is indeed a protein-protein interaction site and, if so, to locate it more precisely. Within a binding interface, individual residues can contribute disproportionately to the binding energy (11), and a recent survey of alanine mutants has confirmed that in general, the contributions of individual amino acid residues to the binding energy is highly uneven, with a small subset of residues providing “hot spots” of binding energy (12). Therefore, we reasoned that for a genuine binding site, alanine substitutions should result in a characteristic pattern of highly variable effects on protein function, where substitutions at residues directly involved in binding result in the greatest loss of activity.

Materials and Methods

Reagents

Most materials for assessing hemolytic activities and protease sensitivities including buffers, purified complement components, sensitized erythrocytes, cobra venom factor (CVF, Naja naja kaouthia) and C5-depleted serum were purchased from Advanced Research Technologies (San Diego, CA). Goat antiserum against human C5 and monoclonal anti-human C5 were from Advanced Research Technologies and Quidel (San Diego, CA), respectively. Peptide inhibition of complement hemolytic activity was assayed using the EZ Complement kit (Dianexid, Miami, FL). Oligonucleotides were from Sigma-Genosys, The Woodlands, TX.

Synthetic peptides were synthesized and reverse phase HPLC purified by the peptide synthesis facility of this Institute. Stock aqueous solutions were prepared at 5 mg/ml and neutralized as necessary with (NH₄)₂HCO₃.

Construction and expression of C5 mutants

Mutants were constructed by altering the human C5 cDNA sequence in pH5.C.D (5) by overlap extension PCR (13), expressed transiently in COS cells cultured in 1% Nutridoma HU medium (Boehringer Mannheim, Indianapolis, IN), and radiolabeled metabolically with [³⁵S]methionine as described (8). Use of the low serum Nutridoma medium was essential for most of the C5 cleavage and binding assays, but not for assessing hemolytic activities.

Analysis of mutants

Recombinant mutant and wt C5 CP hemolytic activities were measured by lysis of Ab-sensitized sheep erythrocytes (EA, Advanced Research Technologies, San Diego, CA) in the presence of C5-depleted serum, and were quantitated by ELISA with a C5-specific mAb as described (8, 9). Immunoprecipitation, SDS-polyacrylamide gel electrophoresis, autoradiography, and densitometry (LKB Ultrascan XL, LKB Instruments, Gaithersburg, MD) were also as described (5, 14). Specific activity measurements were conducted at least in quadruplicate, with two measurements each for independently isolated duplicate mutant clones.

C5 activation by the CVF:Bb convertase was conducted as described (5). After an initial 15-min incubation of CVF with factors B and D, the resulting CVF:Bb convertase was added to 500 µl transfected and radio-labeled COS cell supernatants. For essentially complete activation, we used a final concentration of 35 µg/ml CVF for 1–3 h at 37°C. Milder conditions (15 µg/ml CVF:Bb; 37°C for 30 min) giving 35–45% cleavage of the input C5 were used to increase the sensitivity of experiments in which the susceptibilities of wild-type and mutant C5 were compared. After incubation with CVF:Bb, the reaction mix was centrifuged, the supernatant was collected, and the reaction products were immunoprecipitated and analyzed by gel electrophoresis.

C5 α-chain cleavage by the CP convertase was conducted with convertase-bearing EAC1423 cells. These were prepared by first treating EA at 1 × 10⁷/ml in SGVB (sucrose-gelatin-Veronal buffer; from Advanced Research Technologies) with 25 µg/ml C1 (final concentration) for 20 min at 37°C. The resulting EAC1 cells were washed with SGVB³⁺, resuspended in SGVB³⁺ to 2.5 × 10⁶ cells/ml, and treated with 12 µg/ml C4 for 20 min at 37°C. The product EAC14 cells were washed in SGVB³⁺, resuspended in the same buffer to 1 × 10⁷/ml, and placed on ice. For cleavage reactions, C2 and C3 were added to EAC14 cells to final concentrations of 25 and 90 µg/ml, respectively, and 50-µl aliquots of this mixture, together with 3 µl C6 at 1 mg/ml, were added immediately to 500 µl radio-labeled COS supernatants. The reaction mix was incubated at 30°C for 1 h, then chilled on ice, and centrifuged for 5 min. The supernatant was collected and reaction products were immunoprecipitated with C5-specific antiserum and separated on an SDS-polyacrylamide gel.

C5 cleavage was added to the immunoprecipitated products to facilitate release of the product C5b into solution. In the absence of C6, we found ~55% of the C5b in solution and the remainder associated with erythrocytes, whereas in the presence of C6, ~85% of the C5b was in solution. The amount of C5b associated with erythrocytes was assessed by comparing the amounts of C3 α-chain immunoprecipitated from solution and from a detergent (1% IGEPAL-CA630, Sigma, St. Louis, MO) lysate of the erythrocytes. This tendency of C5b to adhere to erythrocytes is consistent with earlier reports (e.g., Refs. 15 and 16).

Binding of C5b to C6

Radiolabeled C5 in 500 µl COS supernatant was incubated with CVF:Bb (35 µg/ml CVF) as described above in the presence of 25 µg/ml C6 for 3 h at 37°C. The reaction mix was then placed on ice and centrifuged in the cold to pellet any insoluble material. Aliquots of the supernatant were immunoprecipitated with C5 and C6 antiserum specific for either C5 or C6, respectively, and the reaction products were displayed on SDS-polyacrylamide gel. Under these conditions, ~20% of the input C5 coprecipitates (as C5b) with C6. This is essentially identical with the proportion of C5b,6 complex obtained by DiScipio (17) using a sucrose gradient centrifugation assay. Therefore co-immunoprecipitation is quite efficient.

Peptide inhibition of complement hemolytic activity and CP convertase

Inhibition of hemolytic activity was assayed essentially as described (6). Briefly, EA were incubated for 1 h at room temperature with varying amounts of peptides and 0.1% human serum, which gave ~50% lysis of the input erythrocytes in the absence of peptide. After incubation, samples were centrifuged to pellet the intact erythrocytes, and the supernatant assayed for A412. Peptide-inhibitory activities were expressed as the percentage peptide concentration necessary to decrease the serum hemolytic activity to 50% of the control activity in the absence of peptide (I₅₀). Inhibitory activities of the alanine-substituted peptides relative to the parent peptide 5-26a was expressed as the ratio I₅₀/5-26a(I₅₀/5-26a):I₅₀(alanine-substituted peptide).

Peptide inhibition of the CP convertase was assessed by measuring the effect of 180 µM peptide on cleavage of radiolabeled wtC5 in COS supernatants by EAC1423 cells as described above. EAC1423 cells were at 1 × 10⁷/ml and C6 was also present at a final concentration of 5 µg/ml. The reaction mixture was then incubated at 30°C for 30 min. Peptide activity was determined by immunoprecipitation with a C5-specific anti-serum and gel electrophoresis. Under these conditions and in the absence of peptide, 25–50% of the C5α chain was cleaved to the C5α’ chain. Inhibitory activities of the alanine-substituted peptides relative to the parent 5-26a were expressed as % C5α’ (alanine-peptide)/% C5α’ (5-26a), where % C5α’ is determined from the autoradiographic intensities of the C5α and C5α’ bands on SDS-polyacrylamide gels.

Peptide inhibition of the CVF:Bb convertase was measured by incubating radiolabeled wtC5 in COS supernatants with CVF:Bb at 15 µg/ml CVF and
FIGURE 1. Sequences of human C3, C4, C5, and C5 mutants C5/Id26A and C5/Id26B in the region near indel 26 in the C345 family. In the mutant sequences, dashes indicate identity with wtC5 and asterisks indicate deleted residues. Also given are the relative hemolytic activities of C5 and its mutants determined in Ref. 9.

Alanine scan results suggest a binding site near indel 26 of C5

Alanine substitutions were introduced individually at all sites from K1604 to D1622, with the exception of the native alanine residue at position 1606 and the aliphatic I1618 and L1621. All mutants were expressed transiently in COS cells, and in all cases, including indel mutants C5/Id26A and C5/Id26B, expression levels were within 40% of the wt level of 90 ± 20 ng/ml in any individual experiment, as determined by both ELISA and radiolabeling (8, 9).

Fig. 2 shows the hemolytic activities of the 16 alanine mutants relative to wtC5. The site labeled SL gives the activity of the previously described indel mutant C5/Id26A, where S1623 and L1624 were deleted (9). Sites labeled N are the I1618 and L1621 upstream of indel 26 because the sequences of C3, C4, and C5 immediately downstream face of an interaction between C5 and another protein that is essential for complement hemolytic activity. The downstream residues 1610–1615.

Low activity mutants show low sensitivity to the classical pathway C5 convertase

To determine the molecular basis for low activity in the K1610A, F1613A, and F1615A mutants, we tested the ability of the erythrocyte-bound CP C5 convertase to cleave the α-chains of these mutant proteins. We included in these studies the previously characterized mutant C5/Id26B in which residues LQKY were replaced by residues DFWGE as shown in Fig. 1, because this mutant has no detectable hemolytic activity (9).

FIGURE 2. Hemolytic activities relative to wtC5 of alanine replacement mutants in the segment spanning K1604 to L1624. N indicates that alanine mutants at these residues were not constructed. * indicates that this mutant was a deletion mutant with S1623 and L1624 deleted. Open bar marks the native alanine residue. Note that relative hemolytic activity is plotted on a log scale.

180 μM peptide for 30 min at 37°C, followed by immunoprecipitation and gel electrophoresis. Under these conditions, 35–40% of the input C5* hemolytic activities relative to wtC5 of alanine replace-
recognized by the convertase. The data in Fig. 3 and Table I also show that susceptibility to CVF,Bb is not affected by either the alanine scan mutations or the 5-residue substitution in C5/Id26B. In Fig. 3, CVF,Bb cleaved \(~38\%\) of the wtC5α-chain under these conditions. The concentration of CVF,Bb was decreased to give limited digestion in this experiment to increase the chances of detecting any loss of susceptibility to the protease. These results indicate that the putative binding site is recognized by the CP convertase, but not by CVF,Bb. Some differences in recognition by these convertases would be expected because they are distinct proteins, but also because CVF,Bb recognizes both C3 and C5, whereas the CP C5 convertase recognizes C5 exclusively (19).

**Alanine scan mutants show normal binding to C6**

All mutational studies are plagued by the possibility that the engineered mutations cause long range structural changes in the protein that alter protein function without being at the active site itself. The alanine substitutions in K1610, F1613, and F1615, as well as the more extensive 5-residue substitution in C5/Id26B, do not appear to cause long range structural perturbations, because they do not affect either expression of the normal αβ-chain structure by COS cells or activation by CVF,Bb. Nevertheless, long range perturbations have been observed in alanine scan mutants (see, e.g., Ref. 20); therefore, as a further test of the native C5 structure, we tested for the ability to bind C6. Proteolytic activation of C5 to C5b results in the transient display of a binding site for C6 on the tested for the ability to bind C6. Proteolytic activation of C5 to C5b,6 is quite stable and can be isolated by standard biochemical methods when it is formed in solution by CVF,Bb activation of C5 in the presence of C6 (22). We found that it can also be immunoprecipitated by an antisera specific for C5. Fig. 4 shows the results of activating radiolabeled wtC5 and C5/Id26B by CVF,Bb in the presence of C6, and subsequent immunoprecipitation with a C6-specific antisera. For comparison, Fig. 4 also shows the results of parallel experiments in which CVF,Bb was omitted and where a C5-specific antisera was substituted for the C6-specific serum. We tested only C5/Id26B, because it should undergo the most extensive structural perturbations.

The results in Fig. 4 demonstrate that the C6-specific antisera immunoprecipitates identical amounts of wt and mutant C5b. This is strong evidence that the wt and mutant proteins have the same capacity to form the C5b,6 complex. In contrast to the C5-specific antisera, the C6-specific antisera precipitates the α′β-chains of C5b, but not the αβ-chains of C5, and it does so only when C6 is present during activation by CVF,Bb. These results demonstrate that the 5-residue substitution in C5/Id26B, which has no detectable activity, does not interfere with the ability of the activated mutant protein to bind C6. We infer that this is also the case with the doubtless less disruptive alanine scan mutants.

### Indel 26 peptide inhibits the CP convertase

We previously reported (9) that the synthetic peptide MKGEAL QIKYNFS-NH₂, dubbed 5-26a, inhibits complement-hemolytic activity with an ICH₅₀ of 50 μM. This peptide corresponds to C5 residues 1602–1614 and includes K1610 and F1613, but not F1615, in the putative core C5 convertase recognition site, KYN FSF. The mechanism of peptide inhibition is not known, but one simple possibility is that 5-26a is an “interface” peptide, corresponding to part of a protein-protein binding interface, and acting as a competitive inhibitor of that binding reaction (e.g., Ref. 23). If this is the case, our mutagenesis results would predict that the peptide should interfere with C5 activation by the CP, but not by the CVF,Bb convertase. To test this idea, we measured the effect of 5-26a on C5 activation by the CP and CVF,Bb convertases.

Our results in Fig. 5 show that peptide 5-26a inhibits CP convertase cleavage of C5: 50% of the input C5α-chain is cleaved to the α′-chain in the presence of peptide, and this is decreased to 15% in the presence of the peptide. In contrast, the peptide does not affect cleavage by CVF,Bb. The experimental conditions in both experiments were again adjusted to give limited C5 cleavage to maximize any inhibitory effects of the peptide. These results provide strong evidence that 5-26a inhibits complement hemolytic activity by interfering with C5 activation and that it does so by an
interface peptide mechanism, blocking the same interaction between C5 and the CP convertase that is affected by alanine substitutions in the intact protein.

In Fig. 5, the lane showing the effect of 5-26a on cleavage by CVF,Bb has a high background signal that is characteristic of many experiments involving activation by this enzyme. We do not observe this high background with CP convertase activation. Hence it appears to be peculiar to activation in solution by CVF,Bb, where the product C5b may adhere nonspecifically to radiolabeled material in the COS supernatant. This problem was especially severe in this experiment with 5-26a, possibly because of additional nonspecific adhesion of radiolabeled material to peptide aggregates. Centrifugation of the sample after incubation with CVF,Bb eliminated this background but also removed most of the C5 and C5b.

**Ala-substitution of synthetic peptide 5-26a reflects alanine-scanning mutagenesis results**

As an additional test of the idea that peptide 5-26a acts as an interface peptide, we tested the inhibitory activities of alanine substitution variants of 5-26a (MGKEALQIKYNFS-NH2) relative to the parent peptide. The sequence of the substituted region is shown on the abscissa, and the results of alanine substitution at each residue is shown above that residue. Wide, darkly shaded and open bars show C5 hemolytic activities (from Fig. 2), narrow striped bars show the relative inhibitory activities of peptide variants on complement hemolytic activity, and narrow lightly shaded bars show the relative inhibitory effects of peptides at 180 μM on C5 cleavage by the CP C5 convertase; the peptide inhibition results are the average of two measurements.

**FIGURE 6.** Comparison of the hemolytic activities, relative to wtC5, of alanine scan C5 mutants with the inhibitory activities of alanine-substituted variants of 5-26a (MGKEALQIKYNFS-NH2), relative to the parent peptide. The sequence of the substituted region is shown on the abscissa, and the results of alanine substitution at each residue is shown above that residue. Wide, darkly shaded and open bars show C5 hemolytic activities (from Fig. 2), narrow striped bars show the relative inhibitory activities of peptide variants on complement hemolytic activity, and narrow lightly shaded bars show the relative inhibitory effects of peptides at 180 μM on C5 cleavage by the CP C5 convertase; the peptide inhibition results are the average of two measurements.

Discussion

In previous work we suggested that recognition of C3 and C5 by their respective convertases requires interactions at sites distal to the activation cleavage sites in these proteins (4, 5). To find these putative distal sites, we developed a strategy focused on indels in the C345 protein family. Our initial studies yielded a number of candidate protein-protein interaction sites in C3 and C5 (6, 8, 9), and in this report we present evidence that one of these candidate sites, near indel 26 in C5, is a binding site recognized by the classical pathway C5 convertase.

The putative CP C5 convertase binding site is focused on the lysine and both phenylalanine residues in the sequence KYNF, which spans residues 1610–1615 in human pro-C5 (18); its location relative to the convertase cleavage site in the C5 α-chain is illustrated in Fig. 7. This sequence is quite typical of binding interfaces, where lysine and phenylalanine residues are found with moderate frequency (29). Tryptophan, tyrosine, and arginine are the most frequent contributors to binding hot spots (12), and an arginine and several tyrosine residues lie within or very close to the core KYNF (henceforth referred to as KF) sequence. However, as seen in Fig. 2, alanine substitutions at these residues result at most in only modest changes in activity; hence they do not appear to contribute substantially to the binding energy in this case.

Three other features of this putative binding site are notable. 1) The core KYNF sequence contains the potential glycosylation site, NFS (30). However, DiScipio has shown that this site is not
FIGURE 7. Convertase recognition sites in C5. Diagrams of the C5 α- and β-chains are as labeled. The darkly shaded overlapping ellipses represent the C2a and the C3b or C4b subunits of the CP convertase. The notch in C2a represents the protease active site; pockets in C3b/C4b represent hypothetical binding sites for C5. The downward arrow marks the convertase cleavage site. Lines interrupted by S-S are disulfide linkages inferred from those of C3 (43); not all disulfide bridges are shown. The site marked te is the corresponding location of the intramolecular thioester in C3 and C4. Thick gray lines extending from the pockets in C3b/C4b point to interaction sites on C5 with the CP and CVF convertases; the solid line represents the CP convertase interaction at the KF site in the C345C module, dashed lines show proposed interactions. Vertical lines in the α-chain labeled 14 and 15 are the locations of indels 14 and 15.

glycosylated in human C5 (17). 2) The corresponding core sequence in mouse C5 is KHNFSF (31); hence the critical lysine and phenylalanine residues are conserved in the murine protein, but the nonessential tyrosine residue is replaced by histidine. This is consistent with the compatibility of murine C5 with human components in hemolytic assays (32). Finally, the corresponding sequences of C3 and C4, GEPKPNL, and DLEGHP, respectively, diverge substantially from the C5 sequence.

Several independent observations support the idea that the alanine scan data reflect direct disruption of an intermolecular interaction and not an intramolecular conformational change. 1) For all mutants, including the previously described indel mutants C5/Id26A and C5/Id26B, the level of expression and the efficiency of processing of the precursor protein by COS cells (assessed by measuring the relative amounts of precursor and the α- and β-chains of the mature protein) were indistinguishable from those of wtC5. 2) All mutants were efficiently immunoprecipitated by a C5-specific antiserum. 3) All mutants were activated by CVF,Bb with efficiencies equal to wtC5. 4) The most extensively substituted mutant C5/Id26B, with null hemolytic activity, retained the normal activation-dependent ability to bind C6. Representative evidence for these conclusions is given in Figs. 3 and 4 and in Ref. 9. Finally, the observations that peptide 5-26a inhibits complement-hemolytic activity and CP convertase, but not CVF,Bb cleavage of C5 (Fig. 5), and that the effects of alanine substitution of this peptide reflect those of alanine substitution of the intact protein (Fig. 6), constitute strong independent evidence that this region of C5 is a binding site for the CP C5 convertase and that peptide 5-26a is an interface peptide, competing with C5 for the same binding site on the convertase.

The KF site may constitute the entirety of a discrete binding site or it may be one component of a site composed of several non-contiguous segments. In either case, this site is unlikely to be the only binding site for the CP convertase, because C5 binds to both of the noncatalytic subunits, C4b and C3b, in the convertase (16, 33, 34). Our previous indel scanning results suggested that there is another convertase recognition site at indel 15 (near residue 863, 130 residues downstream of the convertase cleavage site at R733), because deletion of residues 863–868 resulted in substantial loss of both hemolytic activity and susceptibility to cleavage by CVF,Bb (9). This idea was confounded, however, by the observation in the same study that a 5-residue insertion at indel 14 (between residues 766 and 767) resulted in a similar loss of sensitivity to CVF,Bb but had absolutely no effect on hemolytic activity (see Fig. 7). We speculated at that time that this apparent inconsistency might be a convertase-specific effect, where the indel 15 region is recognized by both the CP convertase (the CP mediates hemolysis) and CVF,Bb, whereas the region near indel 14 is important only for recognition by CVF,Bb. In this picture (Fig. 7), the CP convertase recognizes regions near indels 15 and 26, whereas CVF,Bb binds near indels 14 and 15. These putative sites may provide separate and distinct binding sites for distinct regions of the convertases as illustrated in Fig. 7, or they may be constituents of the same binding structure. We cannot distinguish between these possibilities, but the disulfide link between C848, just upstream of indel 15, and C1509, just upstream of the C345C module (see Fig. 7) suggests that indel 15 and the C345C domain lie in close proximity in the native structure.

The binding site for the CP C5 convertase lies within the C345C/NTR module of C5

The KF site lies within a cysteine-rich structural module previously referred to as C345C (35) and NTR (36). This ~150-residue-long module was first recognized as a potential structural unit common to the Caenorhabditis elegans UNC-6 protein and C3, C4, and C5 by Ishii et al. (37), who noted for the complement proteins that it is absent in the paralogous α2-macroglobulin. C345C/NTR is also a structural component of netrins, secreted frizzled-related proteins, type 1 procollagen C-proteinase enhancer proteins, and tissue inhibitors of metalloproteinases (TIMPs). However, with the exception of TIMPs, where it is a binding site for the metalloproteinase, and now C5, where it is a binding site for the CP convertase, the functional roles of these modules are unknown (36).

For any protein, the precise placement of indels by multiple sequence alignment programs usually depends on the specific collection of proteins used in the sequence alignment. Therefore, for example, the location of indel 26 varies depending on whether human sequences alone, or both human and mouse C3, C4, and C5 sequences are used in the alignment. In an alignment exclusively of C345/NTR modules from different proteins, the KF sequence lies at an indel situated almost exactly midway between groups of highly conserved residues (36); this reinforces the idea that indels provide good starting points for binding site searches. Among modules from different proteins, the variable regions separating these conserved residues range in size from 6 residues in the TIMPs to 16 residues in human netrin-2 like protein; hence, this segment of the module tolerates substantial length polymorphisms. This segment is quite long in the complement proteins, 15 residues in C5 and 13 residues in C3 and C4 (the 2-residue difference yields indel 26 in our alignment of C3, C4, and C5). The C345C/NTR alignment shows three other regions with unusually large length polymorphisms, two of which harbor indels we designated as 25 and 27 in the C345 family (6, 36). However, only mutations at indel 26 had any effect on C5 activity (9).
Structure of the binding site

Three-dimensional structures of human TIMP-1 and TIMP-2 in complex with metalloproteinase targets have recently been determined by x-ray crystallography and NMR spectroscopy, respectively (38, 39). The common structure of the C345C/NTR domain consists of a five-stranded β barrel and two terminal α helices packed side by side against the barrel face. With regard to our focus on indels to find binding sites, Bányai and Patthy found it noteworthy that gaps (indels) in their multiple alignment of C345C/NTR modules correspond to surface loops in the TIMP-2 structure (36).

The metalloproteinase binding site in this structure is composed of the N terminus of the protein, and the loops connecting β-strand pairs AB, CD, and EF (the β-strand designations are shown in italics to avoid confusion with amino acid residues). The AB β-strand structure in TIMP-2 is an unusually long extended β hairpin that makes extensive contacts with the catalytic domain of the target matrix metalloproteinase (39). Assuming an equivalent structure in C5, residues KYNPSF lie in the loop connecting β strands D and E at the opposite side of module (36). Hence, binding sites can occupy opposite faces of this module. Both D and E strands are much longer in C5 than in the TIMPs and therefore may form an extended β structure similar to AB in TIMP-2. The best β sheet-forming amino acids are threonine, isoleucine, tyrosine, tryptophan, phenylalanine, and valine, and the poorest are proline, glycine, alanine, aspartic acid, and asparagine (40). Therefore, the ALQ1 and RYI1 sequences flanking theKF site are consistent with a β hairpin structure.

The AB hairpin in TIMP-2 forms only a part of the metalloproteinase-binding site. Similarly, the putative DE β-hairpin/loop structure in C5 may be an essential, but not exclusive component of the CP convertase-binding site. In the three-dimensional structure of the C345C/NTR module, the segments containing indels 25 and 27 are on the opposite side of the module and hence would not be expected to take part in convertase binding, consistent with our finding that mutations at these sites do not affect C5 activity (9). The segment closest to the DE loop, and therefore the best candidate for additional interactions with the convertase, is the highly charged loop connecting β strands B and C that includes the sequence EAVAKDSE, which extends from residues 1571 to 1579. This segment includes a single residue indel in the C345 family (an insertion in C4 relative to C3 and C5) that we chose not examine in our initial scan because of its small size (6). In the netrins, the insertion in C4 relative to C3 and C5) that we chose not examine in our initial scan because of its small size (6). In the netrins, the insertion in C4 relative to C3 and C5) that we chose not examine in our initial scan because of its small size (6).

Finally, it is possible that peptide 5-26a itself assumes a β-hairpin structure similar to the putative structure of the corresponding segment in C5. Short linear peptides are present in solution as an ensemble of rapidly interconverting conformations that together usually give the appearance of a random structure (27). For peptide 5-26a, a β hairpin structure may dominate this ensemble. Such a compact, defined structure would be consistent with the effective-ness of this peptide as a complement inhibitor, and also with the unexpected loss of activity of the two variants in which nonessential tyrosine and asparagine residues were replaced by alanine (see Results), because alanine substitutions destabilize the β structure (42).

Circular dichroism spectra (not shown) of 5-26a in PBS at room temperature and at 5°C indicate a random structure, however, and hence the β structure, if it indeed dominates, must be only stable enough to make binding by induced-fit more favorable energetically.

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References