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Active Sites in Complement Component C3 Mapped by Mutations at Indels

Ronald T. Ogata, Rong Ai, and Pamela J. Low

Engineered mutants of human complement component C3 were used to test the idea that sites of length polymorphisms in protein families (indels) can guide a search for protein:protein interaction sites. Sequence changes were introduced at each of the 27 indels in the C3/4/5 protein family, and mutants at 26 indels were expressed by transiently transfected COS cells. Expressed proteins were assayed 1) for concentration, by ELISA and by autoradiography of radiolabeled protein; 2) for classical pathway hemolytic activity; 3) for susceptibility to proteolytic activation by the alternative pathway and cobra venom factor C3 convertases; and 4) for susceptibility to complement factor I in the presence of factor H. Most of the mutations did not appreciably alter expression or activity relative to wild-type C3, consistent with the idea that most indels occur at the protein surface. Mutations at four indels severely damaged C3 functional activity, but did not affect the stability or structure of the protein, as assessed by their effects on expression by COS cells and on susceptibility to cleavage by C3 convertases and factor I. These indels are therefore near functionally important amino acid residues; they represent good candidates for sites of protein:protein interactions. Mutation of the sequence at a fifth indel altered the equilibrium between the latent and reacted C3 conformations, and mutations at 4 other indels substantially decreased both protein activity and expression. The mutants provided an overview of the structural and functional roles played by different parts of C3.


Complement component C3 is an ~190-kDa heterodimer that plays a central role in the activation and regulation of the complement system. In this role, C3 or its fragments interact with at least a dozen other proteins during activation and regulation of complement (reviewed in Ref. 1). A number of sometimes contradictory peptide-binding and site-specific mutagenesis studies have been carried out to locate some of the interactive sites in C3, primarily those recognized by membrane-bound C3 receptors or by proteins that regulate C3 activity (e.g., 2–6).

Our own work has been directed at dissecting the interaction between C3 and C3-specific activating proteases. The overall aim is to discern the structural features of C3 and of the closely related components C4 and C5 that are important for their specific recognition by unique activating proteases. Previous results suggest that recognition of C3 and C5 involves protease binding at sites distal to the cleavage site itself, whereas the C4-specific protease recognizes features in the immediate vicinity of the C4 activation site (7, 8). An undirected search for the putative distal binding sites in C3 is impractical, because it is a large protein with >1600 amino acid residues. Therefore, we recently proposed that a search could be limited by focusing on regions near indels in the protein family consisting of C3, C4, and C5 (9).

Indels are the insertions or deletions of amino acid residues that give rise to length polymorphisms among members of a protein family. They are called indels because an insertion in one member of a family is equivalent to a deletion in another (10). We reasoned that indels are good candidates for protein:protein interaction sites because they usually occur near residues at the protein surface, and usually in coils within loops or reverse turns (11–14), which may be “ideal sites for receptor recognition because they present side chains in a highly accessible arrangement around a compact folding of the peptide backbone” (15).

C3, C4, and C5 have distinct functions and binding specificities, but similarities in their sequences, subunit and precursor structures, protease sensitivities, and other properties suggest that they share very similar three-dimensional structures. Therefore, we speculated that the unique features of each protein might be due, at least in part, to unique sequences at indels, arranged on a common peptide backbone “core.” We also reasoned that a focus on indels might, with appropriate choice of functional assays, provide an effective general approach to mapping other binding sites in C3, not just those recognized by activating proteases, and binding sites in C4 and C5 as well.

While a focus on indels is straightforward conceptually, assessing the involvement of a particular indel in a protein:protein interaction can be problematic. We tried to test the indel approach first by constructing peptides with sequences corresponding to indel-proximal segments of C3, and measuring the effects of these peptides on complement hemolytic and bactericidal activities (9). This strategy assumed that a C3 peptide with a sequence recognized by a C3-binding protein can itself bind to that protein and competitively inhibit C3 binding. If that interaction were necessary for C3 function, then the peptide would block complement activity.

A total of 21 peptides were tested and 4 were found to inhibit both complement hemolytic and bactericidal activities; hence, that study successfully identified 4 peptide inhibitors of complement activity, and 4 potential protein:protein interaction sites in C3. It also provided support for the idea that binding sites lie near indels.

In the present study, we engineered variants of C3 with sequence changes at each indel, and assayed their functional activities and protease sensitivities. This mutational method provides an independent and complementary test of the idea that indels can guide a search for protein:protein interaction sites.
Materials and Methods

Reagents

Most materials for assaying C3 activity and protease sensitivity, including buffers, purified proteins, sensitized erythrocytes, and serum reagents, were purchased from Advanced Research Technologies (San Diego, CA). Goat anti-human C3 antisera and monoclonal anti-C3 were from Advanced Research Technologies and Quidel (Lafayette, CO), respectively. Oligonucleotides were from Genosys Biotechnologies, The Woodlands, TX.

C3 mutant construction

Mutants were constructed by altering the hC3 cDNA sequence in pSV-C3 (3) by overlap extension PCR (16) employing Pfu DNA polymerase (Stratagene, La Jolla, CA). Sequences were confirmed by manually sequencing by standard methods the entire amplified segment after reinsertion into the cDNA. Plasmid DNAs for transfection were isolated with the purification kit from Qiagen (Santa Clarita, CA). In most cases, two independent clones for each mutant were isolated and DNA from each was used in duplicate transfections.

Expression of C3 mutants

Recombinant C3 was obtained from culture supernatants of transiently transfected COS cells. COS cells maintained in DMEM supplemented with 10% FBS were transferred to medium consisting of equal volumes of RPMI 1640 and DMEM supplemented with 1% Nutridoma HU (Boehringer-Mannheim, Indianapolis, IN) 2 days prior to transfection. Transfections were performed by the DEAE-dextran method with 3 μg of plasmid DNA as described (17). Following glycerol shock, the cells were maintained in the RPMI 1640/DMEM/Nutridoma medium. Culture supernatants were collected approximately 72 h after transfection, placed on ice, and assayed immediately for C3 concentration and within 2 h for C3 hemolytic activity as described below. Supernatants were replaced with methionine-free RPMI 1640/DMEM/Nutridoma medium containing [35S]methionine, and cell cultures were incubated an additional 7 h to obtain radiolabeled proteins. These supernatants were chilled and tested within 30 min for susceptibility of radiolabeled proteins to the alternative pathway (AP) and cobra venom factor (CVF) C3 convertases, and to factor I in the presence of factor H. All transfections included wild-type pSV-C3 as a standard. Up to 16 transfections (15 mutants + wild-type [wtC3]) were carried out in duplicate simultaneously.

ELISA quantitation of rC3

C3 concentrations in transfected COS supernatants were measured by a competition ELISA. C3-bearing microtiter plates were prepared by incubating 100 μl/well overnight at 4°C, followed by multiple washing with water, treating with 200 μl of PBS containing 1% BSA/0.2% sodium azide for 1h at 37°C, and washing again with a solution of COS supernatant and monoclonal anti-human C3 (90 μl supernatant and 10 μl of 80 ng/ml Ab (in PBS containing 1% BSA/0.5% Tween-200/0.2% sodium azide) previously incubated at 37°C for 30 min) was added to treated wells and incubated at 37°C. After 2 h the solution was removed and the assay completed by washing with water, incubating with biotin-conjugated goat anti-mouse-Ig Ab (100 μl/well at 0.8 μg/μl from Zymed, San Francisco, CA) for 2 h at 37°C, followed by washing with water and incubating with 3 μg/ml streptavidin-conjugated alkaline phosphatase (Zymed) at 100 μl/well for 30 min at 37°C, again washing with water, and then incubating with 100 μl of 40 mg/ml phenolphthalein monophosphate (di(cyclohexylammonium) salt; Sigma, St. Louis, MO) for 30 min at room temperature and, finally, measuring the absorbance at 540 nm. C3 concentrations were determined by comparison with reference samples of C3 purified from serum (serC3) diluted into mock-transfected COS culture medium.

C3 hemolytic activity

Activity was measured by a hemolytic assay. A total of 50 μl of transfected COS supernatant were added to a solution consisting of 150 μl GVB + (Veronal-buffered saline with 0.15 mM CaCl2, 0.5 mM MgCl2, and 0.1% gelatin), 100 μl Ab-sensitized SRBC (EA) at 1.5 × 108 cells/ml and 5 or 10 μl C3-depleted serum. After incubation at 37°C for 30 min, the sample was chilled on ice, diluted with 0.5 ml of GVB + , centrifuged to pellet intact erythrocytes, and the absorbance at 412 nm was measured to assess lysis. A reference plot of percentage of lysis vs C3 concentration was constructed using dilutions (into mock-transfected culture supernatant) of serC3, and the activities of the rC3 samples were determined from this plot, with activities expressed as the concentrations of C3 giving equivalent lysis. Activity (in nanograms per milliliter of C3) divided by the concentration of C3 in the culture supernatant (in nanograms per milliliter) determined from the ELISA was defined as the fractional specific activity. By this definition, the fractional specific activity of the serC3 standard is 1.0. Relative specific activity is defined as the fractional specific activity divided by the fractional specific activity of wtC3 from the same set of transfections.

Proteolysis of radiolabeled C3

C3 α-chain cleavage by the AP and CVF convertases, immunoprecipitation, SDS gel electrophoresis, and autoradiography were performed as described (8, 18). The extent of proteolysis was measured by densitometry (LKB Ultrascan XL) of autoradiograms with relative α-chain cleavage defined as (α′/α)/(α′/α0), where α′ and α are the autoradiographic intensities of the respective mutant subunits, and α′0 and α0 are the intensities of the wild-type subunits from a coincident transfection and displayed on the same gel. Under the reaction conditions used, 30 to 50% of the wild-type α-chain was converted to α′ by the AP convertase, with 55 to 65% cleavage by the CVF convertase. AP cleavage was measured at least twice, using supernatants from independent transfections, and CVF cleavage was measured once (twice for Id3, Id13, and Id14).

Proteolysis by factor I in the presence of factor H was carried out by adding 0.4 μg factor I and 2 μg factor H to 100 μl of COS supernatant and incubating for 30 min at 37°C. The sample was then immunoprecipitated and fractionated by SDS-PAGE as described above. The extent of cleavage was assessed by densitometry of the α- and β-chains in the autoradiograms. A crude measure of the percentage of C3(H2O) in the sample was obtained by assuming that the loss in α-chain intensity after I/H treatment is equal to 0.65 times the gain in β-chain intensity; 0.65 is the fraction of α-chain molecules that are in the α76K peptide, which extends from the α-chain N terminus to Arg1281. With this assumption, the percentage of C3(H2O) was determined from the ELISA was defined as the fractional specific activity. By this definition, the fractional specific activity of the serC3 standard is 1.0. Relative specific activity is defined as the fractional specific activity divided by the fractional specific activity of wtC3 from the same set of transfections.

Results and Discussion

Design and sequences of indel mutants

Indel mutations were designed to reverse the deletion or insertion in C3 relative to C4 or C5. In some cases, we also made somewhat arbitrary substitutions of a few amino acid residues. This strategy is illustrated below for indels 1 and 3, where the indel-proximal linear sequence of the C3 precursor, pro-C3, as well as the locations of significant structural features in C3 and C4. A complete sequence alignment of human and murine C3, C4, and C5, showing precise indel locations, is given in Reference 9.

<table>
<thead>
<tr>
<th>Indel 1</th>
<th>Indel 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>hC3:</td>
<td>hC3:</td>
</tr>
<tr>
<td>LEANAQ---GDPVFVT</td>
<td>NPEGDPVFQOSLLSQQ</td>
</tr>
<tr>
<td>hC4:</td>
<td>hC4:</td>
</tr>
<tr>
<td>VQLQDPFPQEQVKKSVF</td>
<td>RSNGLKRK---KEV KYM</td>
</tr>
<tr>
<td>hC5:</td>
<td>hC5:</td>
</tr>
<tr>
<td>IQYQVT---SAFADITIS</td>
<td>DFF-EHEV---DVNEYEIR</td>
</tr>
<tr>
<td>hC3/M:</td>
<td>hC3/M:</td>
</tr>
<tr>
<td>LEANAQ---GDPVFVT</td>
<td>NPEGDPVFQOSLLSQQ</td>
</tr>
</tbody>
</table>

A total of 42 mutations were constructed by altering the sequence of pSV-C3 (3) at each of the 27 indels in the C3/4/5 family. Figure 1 shows the sequences of the mutants aligned with the wtC3 sequence. Multiple mutants were constructed at some indels to compare the effects of different mutations at the same indel.

Figure 2 shows the approximate locations of each indel in the linear sequence of the C3 precursor, pro-C3, as well as the locations of significant structural features in C3 and C4. A complete sequence alignment of human and murine C3, C4, and C5, showing precise indel locations, is given in Reference 9.
Expression of indel mutants

All assays were carried out directly on supernatants from COS cells transiently transfected with pSVC3 or its engineered variants. The concentration of C3 in culture supernatants was measured by ELISA using an anti-C3c mAb, and by autoradiographic quantitation of the [35 S]methionine-radiolabeled C3 α-chain after immunoprecipitation with a polyclonal anti-C3 Ab and gel electrophoresis.

Figure 3 shows representative results of immunoprecipitation and gel electrophoresis of radiolabeled recombinant wtC3 and two mutant C3 proteins. Note that the immunoprecipitated products contain the 115-kDa and 75-kDa α- and β-chains, respectively, of the mature C3 heterodimer, as well as a substantial amount of the 190-kDa biosynthetic precursor, pro-C3.

The two independent assays show excellent agreement

Figure 4a shows the expression profile of C3 indel mutants given as the amount of protein relative to wtC3 produced in a coincident transfection. As measured by ELISA, the concentration of wtC3 over 10 transfections ranged from 80 to 250 ng/ml, with an average of 140 ± 40 ng/ml. Most of the mutants were expressed at levels comparable (within a factor of 2) to wtC3, but mutants at indels 4, 8, 9, 11, and 26 were expressed at 20 to 30% of the wild-type level, mutant Id3 was at about 10% of the wild-type level, and Id10 gave no detectable product. The lower levels of expression presumably reflect flaws in the maturation, secretion, and/or stability of the mutant proteins.

The near identical profiles of the two assays are significant for two reasons. First, they provide assurance that the methods are valid. We had anticipated that the ELISA might overestimate C3 levels because COS cells secrete both mature C3 and pro-C3, and we estimated from densitometry that pro-C3 represents 30 to 40% of the total C3 protein. Therefore, if the mAb used in the ELISA reacts with pro-C3, that assay should overestimate the amount of C3 in the supernatant by about 50%; however, the ELISA and radiolabel results do not consistently differ, even by this relatively small amount. This is probably because the proportion of precursor was relatively constant for all recombinant proteins, resulting in a
systematic error in the ELISA that was largely eliminated by expressing mutant C3 concentrations as a fraction of the wild-type level. It is also possible, but less likely, that the C3c mAb does not recognize pro-C3. The radiolabeled indel 10 mutant showed no detectable α-chain, but a small amount of the precursor, which may account for the low level, about 3% of the wtC3 level, detected by the ELISA.

The similarity between the ELISA and radiolabel profiles is also significant because it indicates that none of the mutations affected recognition by the anti-C3c mAb used in the ELISA. Since we expected that the indel approach would provide a broad survey of surface sites on C3, we anticipated that one or more of the mutations would damage the monoclonal binding site, resulting in a much lower C3 concentration measured by the ELISA than by the polyclonal-dependent radiolabel assay for the same supernatant. Mutants altered at indels 1 and 2 show some of this expected behavior, but the difference is rather small, only about twofold.

**Hemolytic activities of indel mutants**

Figure 4b gives the specific hemolytic activities of the indel mutants expressed as activity relative to wtC3. As described in Materials and Methods, relative specific activity is the mutant specific activity divided by the specific activity of wtC3 in a parallel transfection. Recombinant wtC3 from COS cells had about half the specific activity of C3 purified from human serum (serC3); ranging over 10 transfections from 30% to 80%, with an average of 50 \pm 16% of the serC3 specific activity. This low specific activity of recombinant wtC3 can be accounted for by the contribution of pro-C3 to the ELISA, which leads to an overestimate of the wtC3 concentration, and to inactivation of the mature protein due to hydrolysis of the internal thioester. As described previously, densitometry of immunoprecipitated products indicates that pro-C3 is 30 to 40% of the total C3; and as described in detail below, we estimate that 20% of mature recombinant wtC3 is hydrolyzed. Together these two factors indicate that only 50% of the total Ag in COS supernatants is mature, active wtC3, in agreement with the specific activity measurement.

The results in Figure 4b show that the indel mutations cause graded changes in hemolytic activities instead of simple all-or-none effects. This makes identification of protein-binding sites more equivocal, but may provide a more subtle view of the interrelated effects mutations have on C3 structural stability, biosynthesis, and activity. Indeed, the expression, activity, and protease cleavage profiles shown in Figure 4 appear to be sensitive indicators of the varied roles played by indel-proximal residues—most presumably on the C3 surface. The results in Figure 4b may also give a more realistic view of the structure of binding sites. For example, moderate decreases in activity may be due to alteration of only one part of a binding site that is made up of multiple non-contiguous segments of C3.

For many of the mutants, low activity correlates with low expression. For these, the mutations must interfere with the folding and/or stability of the protein, resulting in faulty maturation and/or secretion, and low activity. Dissecting structure-function relationships is especially problematic in these cases. Expression level and activity are not always linked, however, as the mutations at indels 1, 2, and 9 give proteins with 30 to 40% of the wild-type level of expression but normal specific activities, whereas mutants Id14 and Id17 show little activity but near-normal levels of expression.

In spite of the graded responses to indel mutations, the data in Figure 4b clearly indicate that regions of C3 proximal to indels 3, 4, 8, 14, 17, 21, 24, and 26 are important for lytic activity, and perhaps regions proximal to indels 15, 18, 20, 22, and 23 as well. It is difficult to assess the role of indel 10 from these data, but it seems certain that this region is essential for C3 stability or biosynthesis. An interesting feature of this list of potentially important sites is that only indel 20 is in the C3d fragment, while indel 17 is in the C3g fragment, and the remainder are in C3c (Fig. 2). The relative scarcity of indels in the C3d region suggests that this region is largely buried in the native structure—a view that is consistent with the observation that many binding sites for receptors and regulatory proteins in the C3 α-chain are accessible only after a conformational change associated with C3 activation (see Ref. 1).

**Activation of indel mutants by the AP and CVF C3 convertases**

C3 is activated by proteolytic cleavage of the C3 α-chain, yielding an \(\sim 8\)-kDa C3a peptide, and C3b, with an \(\sim 107\)-kDa α′-chain. C3
indel mutants were tested for susceptibility to activation by the fluid-phase AP and CVF C3 convertases to identify binding sites important for recognition by these proteases and to help define the mechanisms responsible for decreased hemolytic activity of the mutants. Figure 3 shows representative results of AP and CVF convertase treatments of wtC3 and mutants Id13 and Id4B. These results do not show the previously observed secondary cleavage of the \( \alpha \)-chain that is caused by bovine factor I in the culture medium (4) because transfections were carried out in a serum-free medium. Figure 4 summarizes the relative sensitivities of the C3 indel mutants to the AP and CVF convertases. The two enzymes should have slightly different recognition requirements, because the former is specific for C3 whereas the latter recognizes both C3 and C5 (19, 20). The results show only a fivefold difference at indel 13, however, which may reflect their resistance to convertase activation. Some resistance to AP convertase is apparently not adequate to cause decreased hemolytic activity, however, since Id13 also shows resistance to AP convertase cleavage (although not CVF convertase), but has essentially normal hemolytic activity. The precise relationship between the convertase cleavage and hemolytic activity results is uncertain, however, because hemolytic activity is mediated by the classical pathway (CP) C3 convertase, which is distinct from both the AP and CVF convertases. A direct comparison was not made because the CP convertase is a labile, membrane-bound enzyme that is difficult to use in this type of broad-scale screen, and an AP hemolytic assay requires higher levels of C3 than were available from the COS expression system.

**What is the mechanism of resistance to C3 convertase?**

Mutations can interfere with C3 convertase in at least two ways: directly, by altering the amino acid sequence at a convertase-binding site, or indirectly, by changing the overall conformation of C3. As represented in Figure 5, proteolytic activation of C3 to C3b, by convertase cleavage of the \( \alpha \)-chain, results in a conformational change from the latent form to the C3b form; these have been designated T and R, respectively, after the classical designations of allosteric conformers (21). The shift from T to R leads to reaction...
of an intramolecular thioester with nucleophiles including water (reviewed in Ref. 22); it renders C3b susceptible to breakdown by the regulatory protease factor I, and active as an auxiliary component of the CP C5 convertase, and the AP C3 and C5 convertases (reviewed in Ref. 23).

Reactions of the thioester with water occur even in the absence of convertase cleavage, giving a hydrolyzed inactive form of C3, designated C3(H2O). This species appears to have the R conformation of C3b, as it is sensitive to factor I, and can substitute for C3b in the AP C3 and C5 convertases. C3(H2O) is also a poor substrate for C3 convertase (24), and hence the convertase shows strong preference for the T form. A mutation that destabilizes the T conformation could inhibit convertase cleavage by inducing a shift to the R form, with concomitant hydration of the thioester. Conversely, any mutant showing normal sensitivity to the C3 convertases should be in the T conformation.

To test the possibility that mutants Id3, Id4, and Id14 have the R conformation, and to assess in general the extent of hydrolysis of rC3 from COS cells, we initially tried to assay for presence of the thioester by autolytic fragmentation (25). This reaction was inefficient in our hands and the results were inconclusive. Therefore, we tested for the R conformation by measuring proteolysis by complement factor I in the presence of factor H (I/H treatment). R form C3(H2O), but not the latent T form is digested under these conditions, yielding ~76 kDa and ~40 kDa peptides, which are the major N- and C-terminal products, respectively, of α-chain scission at Arg1281 and Arg1298 (24, 26). The numbering used is according to DeBrujin and Fey (27), after subtraction of the signal peptide but with retention of the tetra-Arg segment at the βα-subunit junction of pro-C3 that is also absent in mature C3.

Figure 6 shows representative results of I/H treatment of recombinant wtC3 and 3 indel mutants in COS supernatants. These supernatants had not undergone any prior treatment, so any digestion by I/H treatment should reflect the presence of C3(H2O) in the sample. For wtC3 and Id8, there is some loss of intensity of the α-chain band, a slight increase in intensity at the β-chain band, and the appearance of peptides at M, 150 kDa and M, 40 kDa. These changes are expected for factor I proteolysis of the C3(H2O) α-chain and of pro-C3, where the M, 40 kDa fragment is the α-chain C-terminal fragment, the increased intensity at the β-chain position (75 kDa) is due to the comigrating ~76 kDa α-chain N-terminal fragment, and the M, ~150 kDa peptide is the cleavage product of pro-C3 that is composed of the 76 kDa N-terminal α-chain peptide linked to the β-chain. The results with Id13 and Id14 are similar, except that the mutations alter the mobilities of the α-chain N-terminal fragments enough to resolve them from the β-chain. The amount of C3(H2O) in the sample was determined from the loss of α-chain intensity and the accompanying increase in intensity at the β-chain position. The intensity of the band from the 40 kDa product could not be used because this peptide is released on cleavage of both mature and pro-C3.

Id14 shows a high proportion of the R conformation

Table I summarizes the proportion of C3(H2O) in supernatants from transfections with wtC3 and selected mutants, measured by I/H treatment. As mentioned earlier, we estimate from this analysis that for the average transfection, about 20% of the recombinant wtC3 in COS supernatants is C3(H2O). This assay indicates that mutant Id14 has an exceptionally high proportion of the hydrolyzed form, about 70%. This suggests that the mutation at indel 14 causes resistance to convertase activation by shifting the conformational equilibrium of C3 toward the R form. This does not appear to be the case with either Id3 or Id4, the two other mutants

Table 1. Percentage of hydrolyzed C3, C3(H2O), in COS supernatants; estimated by I/H treatment

<table>
<thead>
<tr>
<th>Mutant</th>
<th>% C3(H2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>21 ± 7</td>
</tr>
<tr>
<td>Id3</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>Id4</td>
<td>21 ± 0.1</td>
</tr>
<tr>
<td>Id5</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Id7</td>
<td>22 ± 9</td>
</tr>
<tr>
<td>Id8</td>
<td>32 ± 14</td>
</tr>
<tr>
<td>Id11Δ2L</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>Id13</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Id14</td>
<td>70 ± 4</td>
</tr>
<tr>
<td>Id15</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>Id16</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>Id17</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>Id20</td>
<td>42 ± 1</td>
</tr>
<tr>
<td>Id21A</td>
<td>22 ± 10</td>
</tr>
<tr>
<td>Id22</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>Id23ΔL</td>
<td>17 ± 0.5</td>
</tr>
<tr>
<td>Id24</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>Id26</td>
<td>22 ± 4</td>
</tr>
</tbody>
</table>

Results are the average of two measurements.
displaying resistance to the AP convertase. Of the other mutants showing decreased hemolytic activity, Id8, Id15, and Id17 had a somewhat higher proportion of the R form, and Id24, a lower amount. The differences are only large enough to encourage speculation and further study, however, finally, the precursors that were very sensitive to the AP convertase (suggesting T form stabilization), including pro-Id3, pro-Id4, pro-Id8, pro-Id9, and pro-Id26, did not show unusual resistance to I/H treatment. Therefore the conformations of the precursor and mature proteins may not be comparable.

Mutations do not hinder recognition by factors I and H

With the exception of Id24, all of the mutants tested by I/H treatment had levels of the R form that were at least as high as wtC3. This suggests that none of the mutations affected recognition by I or H. This is especially significant for Id14 in which residues 759–766 are deleted, because previous studies have identified this region of C3, in particular residues 745–754, as a binding site for factor H (2, 28). Digestion of Id14 (as well as two other indel 14 mutants described below) requires both factors H and I (data not shown). The relatively large mutation at indel 20, an insertion of 6-amino acid residues between residues 1200 and 1201, lies within another reported factor H binding site (29), but also does not affect I/H cleavage. Mutant Id24 alone showed a lower than wild-type level of C3(H2O); additional work will be necessary to determine if this is indeed due to lower levels of the hydrolyzed form, to reduced sensitivity to I/H treatment, or to experimental artifacts.

Alternative mutations at indels

A potential pitfall in testing the indel approach by site-directed mutations is the shortcoming shared by all mutagenesis studies: the possibility that the particular mutation selected might give a misleading signal. An overly conservative change may not be adequate to disrupt a binding site, and hence give a false-negative result, whereas an overly large mutation may cause long-range perturbations of the protein structure that give a false-positive result, or a nonexpressing protein. In either case, time and effort are wasted on construction and testing of the mutant. This problem is amplified with large proteins having many indels like C3, where it is important to minimize the number of mutants tested at each indel. To explore this problem, we constructed additional mutants at selected indels in order to assess the effects of different mutations at the same indel and also to confirm and define further some of the candidate binding sites in C3.

Additional mutants at indels 11, 14, and 23 were constructed because the previous peptide study (9) suggested that these were sites of protein–protein interactions important for C3 activity. Indels 3, 4, and 26 were selected because the present results identified them as potential binding sites. A second mutation at indel 2 was constructed to test the combined effect of substitutions and a smaller deletion at a random indel.

Mutant Id21B was constructed to test the possibility that it might give the heterotrimer structure characteristic of C4; this mutant has an insertion of an ArgArgArgArg sequence at a site corresponding to the α3-subunit junction in pro-C4. SDS-polyacrylamide gels (not shown) did reveal a small amount of peptides at Mr ~ 80 kDa and 34 kDa unique to this mutant, corresponding to those expected for cleavage at the tetra-arginine sequence. Hence, there appeared to be some biosynthetic processing at this site; but the low proportion of these peptides indicated that tetra-Arg sequence alone was insufficient to drive the process. Unfortunately, there was not enough of the heterotrimer to examine its properties.

The sequences of the secondary mutants are given in Figure 1, and their properties are listed in Table II. These data show that mutants at the same indel usually share similar properties. For example, the two quite large and very different mutations at indel 2 give almost indistinguishable proteins, both very similar to wtC3. Hence this region of C3 appears to be structurally very adaptable and unimportant functionally. Similarly, all indel 21 and 26 mutants show low activity and near normal expression and convertase sensitivity, while all indel 14 mutants show normal expression, low activity, low convertase sensitivity, and high levels of C3(H2O). Therefore, the secondary mutants confirm many of the indications presented by the initial mutants, and conversely, the initial mutants provided a good general indicator of the effects of mutations at that indel.

The only apparent exceptions to this are at indels 4 and 23, where there are obvious differences in the activities and convertase sensitivities of the individual mutants. Even in these cases there are similarities, however. At indel 4, both insertion mutants showed reduced AP convertase sensitivity, and all mutants showed reduced expression. At indel 23, two mutants show near-identical properties, while expression only of the third is essentially nil. Indel 3 may also be an exception, but Id3A and Id3B, which show normal activity, both involve amino acid substitutions, not a deletion as in Id3; hence their differences may testify to the effectiveness of the reverse insertion/deletion mutagenesis strategy and perhaps to the importance of natural length polymorphisms in protein function.

The series of mutants at indel 11 also differ in their properties, with mutant Id11Δ2L showing much higher activity and expression than the other indel 11 mutants. However, mutant Id11Δ3R was the initial mutant constructed at this indel, according to the insertion/deletion strategy, and the others were constructed only after finding that this mutant gave essentially no expression. Therefore, although the indel 11 mutants show varied properties, Id11Δ3R did provide representative initial information, since mutants Id11Δ3L and Id11Δ2M also show poor expression, especially by the radiolabel assay.
The potential binding sites on C3 identified by indel mutations

The present work has assumed that because indels occur at the protein surface, mutations at indels might damage a protein:protein interaction site and therefore reveal the locations of such interaction sites. Table III lists the C3 mutations causing reduced activities and their locations in the C3 amino acid sequence. Indel 11 was included because of the mixed results for different mutants at this indel (see Table II). On the basis of our original assumption, these are potential sites of protein:protein interactions. The previous peptide study (9) suggested binding sites at indels 11, 14, 22, and 23; hence there is some agreement between the mutational and peptide methods.

Indels 3, 4, 8, and 11 are in the β-chain (see Fig. 2). With the exception of our peptide study, none of the regions surrounding these sites has previously been suggested as a binding site in C3; however, indel 8 is only 10 amino acid residues upstream of the R→W substitution in the inactive C4A6 allotype of human C4. The C4A6 mutation and other engineered substitutions near this site impair the ability of C4b to bind to C5 in the CP C5 convertase (30, 31). A similarly placed binding site in C3 would support the idea that C3, C4, and C5 share similar “core” structures and that their specific properties are due to distinct surface residues decorating this common core at corresponding sites.

Within the α-chain, several of the potential binding sites have been identified previously as important for binding to other proteins or lie near significant structural features. For example, indels 13 and 14 and the sequence intervening (see Fig. 1) has previously been identified as harboring binding sites for factor B, and several complement receptors and control proteins (2, 4, 6); none of these proteins would be expected to be involved in the C3 hemolytic activity assay here, however. Indel 14 is also near the sequence of peptide P-13, which was previously found to inhibit complement (9).

Indel 17 has not previously been associated with a potential binding site, but it lies only 20 residues upstream of the internal thioester in C3 and so this region may be important in the formation of the intermolecular ester linkage of C3b to C4b and C3b in the classical and AP C5 convertases, respectively (32, 33). Similarly, regions near indels 20 and 21 have not been implicated as binding sites in C3, but they correspond (9) to regions with functions unique to C4: the indel 20 segment of C4 cross-links to C3b in the CP C5 convertase (32), and the indel 21 region includes the tetra-Arg sequence at the αγ-subunit junction in pro-C4 (34), as well as residues at the C terminus of the C4 α-chain that are usually absent in the circulating protein (35).

The only evidence that regions surrounding indels 22 and 23 are involved in C3-binding functions comes from our peptide work (9). While indel 23 lies within a few residues of a putative binding site for properdin (29), this is not likely to be relevant, since properdin is important for AP but not CP function. The remaining candidates, indels 15, 18, 24, and 26, have not previously been reported to be associated with binding activities. It may be significant, however, that indel 24 lies within 20 residues of 5 cysteine residues that are totally conserved in human and murine C3, C4, and C5 (9). In the case of indel 26, it is quite striking that very different mutations at sites 11 residues apart cause very similar effects, suggesting that this entire region plays an essential part in C3 function.

Are these all genuine binding sites in C3?

Are all of the indel sites listed in Table III involved in intermolecular interactions? In order to distinguish among these candidates, the mutants were grouped into six types according to their specific properties are due to distinct surface residues decorating this common core at corresponding sites. Within the α-chain, several of the potential binding sites have been identified previously as important for binding to other proteins or lie near significant structural features. For example, indels 13 and 14 and the sequence intervening (see Fig. 1) has previously been identified as harboring binding sites for factor B, and several complement receptors and control proteins (2, 4, 6); none of these proteins would be expected to be involved in the C3 hemolytic activity assay here, however. Indel 14 is also near the sequence of peptide P-13, which was previously found to inhibit complement (9).

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to give mutants that are poorly expressed as well as functionally challenged.

Within this view, the indels in the type 5 mutants with normal expression and low activities (indels 17, 21, 24, and 26) are the best candidate sites for C3:protein interactions that are important in complement activation. Indels in the type 6 mutants with lower than normal activities (indels 15, 18, 20, 22, and 23) are also possible. They gave smaller effects on activity, but residues flanking these indels may form parts of a binding cavity composed of multiple noncontiguous regions; this might explain the surprisingly large number of indels identified as potential binding sites. The lone type 4 mutant at indel 14 also has low activity and normal expression, but the low activity can be explained by the observation that this mutant is predominantly in the C3b-like form, indicating that the region near indel 14 is important for maintaining the latent conformation of C3. The indels altered in the type 1 through 3 mutants cannot be ruled out as binding sites, but the poor or nil expression in COS cells indicates that these sites are at least in part important for the biosynthesis and/or structural stability of C3. In addition, the specific activities of the the type 2 and 3 mutants had high uncertainties due to the low expression levels.

**Have we found all of the CP-binding sites in C3?**

These studies have identified a number of indel-proximal sites in C3 with properties characteristic of binding sites important for CP hemolytic activity. Are there others? In general, a binding site might have been missed either because indel locations do not or only partially correlate with the locations of binding sites, or because as discussed earlier, an inappropriate choice of indel mutation resulted in a false-negative result. Our inability to locate the epitope for the anti-C3c mAb suggests that binding sites can be overlooked, but this may be a special case if, for example, the mAb is directed at a carbohydrate epitope. The two known sites of glycosylation in C3 lie 9 and 15 residues away from the mutations introduced at indels 2 and 16, respectively (27); this may be distant enough to allow unobstructed mAb binding.

We tested our mutants only for CP hemolytic activity, and hence it would be of interest to characterize further the activities of the indel mutants in the alternative pathway, and in binding to complement control proteins and receptors. The present results indicate that none of the mutations affect recognition by control proteins factors H and I. However, these control proteins and the complement receptors do not bind to the latent or T form of the protein. Hence, their binding sites may be sequestered in the “core” structure of the latent protein, and as such may not lie near indels because the core does not tolerate insertions and deletions.

**Summary**

The present results indicate that indels can be useful guides in a general search for binding sites in a protein sequence, at least for the C3/4/5 protein family, since they revealed four regions of C3 (at indels 17, 21, 24, and 26) in which mutations severely damaged activity without affecting protein expression or conformation; these are the effects expected for mutations that alter only an important external binding site. Mutations at 5 other indels (15, 18, 20, 22, 23) similarly did not affect expression or conformation, but had smaller effects on activity. These mutational results showed some agreement with previous peptide inhibition results, which suggested binding sites at indels 11, 14, 22, and 23, but the molecular mechanisms responsible for peptide inhibition remain to be elucidated. An interesting general feature of the mutational results is that all of the mutants displayed characteristic changes in expression, hemolytic activity, and protease sensitivity; hence the results provided an overview of the roles that indel-proximal regions play in the structure and function of C3.

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**References**