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Mutation of recombinant complement component C9 reveals the significance of the N-terminal region for polymerization

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SUMMARY

Complement component C9 binds to C5b-8 sites on target cells and polymerizes to form the membrane attack complex (MAC). The aim of the work reported here was to discover which region within C9 was responsible for protecting the globular protein against self-polymerization. Computer prediction modelling highlighted the domain at the N-terminus of C9, which was then investigated by site-directed mutagenesis. The mutated proteins were expressed using insect cells infected with baculovirus. Removal of 16, 20 or 23 amino acids at the N-terminus of C9 resulted in inactivation due to self-polymerization. In contrast, removal of 4, 8 or 12 amino acids resulted in a C9 that did not polymerize spontaneously, had two to threefold enhanced lytic activity on erythrocytes, and had increased binding to C5b-8 sites on rat neutrophils. These results suggest that the domain within the first 16 amino acids at the N-terminus of C9 is crucial in preventing the self-polymerization of the globular protein. We have also found that C9 contains a motif (27WSEWS31) common to a family of cytokine receptors that is similar to a tryptophan-rich motif (WEWWR) of the membrane pore formers, thiol-activated cytolysins. Mutation of this motif in C9 resulted in polymerized protein, consistent with this site keeping the N-terminus in a protected conformation and preventing premature self-polymerization.

INTRODUCTION

Human complement component C9, a glycoprotein of 538 amino acids and molecular weight on reduced sodium docecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of 71 000 MW[1] is the terminal component of the complement pathway and is required for lysis of cells.[2] Globular C9 interacts with target membranes by binding to C5b-8, followed by a sequence of thresholds[3] involving insertion, aggregation and polymerization. No three-dimensional structure is available for C9, either native or within the membrane attack complex (MAC). However, initial computer algorithms identified five domains in C9 with similarity to other proteins.[1] These included a thrombospondin type I domain (residues 21–77), a low density lipoprotein (LDL) receptor class A domain (residues 78–118), a hinge region (residues 226–267), two amphipathic α-helices that form the membrane-spanning domain (residues 293–334), and an EGF precursor domain (residues 487–521). C9 can aggregate and self-polymerize *in vitro*, especially in the presence of divalent cations such as Zn^{2+} [4,5,6] involving cross-linking of S–H groups. Human C9 has 24 cysteines which are all disulphide bonded. Formation of the complete ring polymer is irreversible and it cannot be disaggregated into monomeric C9.[7] This polymerization of C9 plays a key role in the formation of the MAC, which nucleated cells are able to remove by vesiculation and/or endocytosis.[8,9] A key issue is what mechanism is responsible for signalling formation of the C9 polymer in the MAC.

Our previous work suggested a role for the N-terminal region of C9 in polymerization.[10] Furthermore, an antibody to residues 19–28 of C9[11] bound and inhibited polymerization of C9 as the MAC forms, but did not bind free C9, C9 polymer or the complete MAC. This inferred that the N-terminal region was hidden in the globular protein, but became exposed as the protein unfolded to form the MAC.

To investigate whether the N-terminus of C9 was responsible for protection of globular C9 against self-polymerization, we first analysed this region in detail using computer prediction software for indication of the secondary structure and charge distribution, and database searching for sequence similarity to motifs in other membrane proteins. Recombinant C9s with a mutated or deleted N-terminus were then expressed in insect cells using baculovirus infection. This system was necessary to produce active, correctly folded and glycosylated C9 that did not self-polymerize.[10,12] Three criteria were used to investigate

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Abbreviations: HIS, C-terminal polyhistidine tag; KRH, Krebs Hepes buffer (120 mM NaCl, 25 mM Hepes, 4·8 mM KCl, 1·2 mM KH_2PO_4, 1·2 mM $MgSO_4$ and 1·3 mM $CaCl_2$) adjusted to pH 7·4 with NaOH; KRHA buffer, KRH buffer and 0·1% bovine serum albumin.

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the effect of mutation on polymerization: (a) the ability to self-polymerize; (b) the rate of haemolysis, which depends on polymerization of C9 within the MAC; and (c) binding of the mutants to C5b-8 sites on cells.

The results reported here showed that a domain within the first 16 amino acids of the N-terminus of C9 is crucial in preventing self-polymerization of the globular C9 protein.

MATERIALS AND METHODS

Materials

Magic polymerase chain reaction (PCR)-prep was obtained from Promega Ltd (Southampton, UK), Geneclean from Bio 101 Inc. (La Jolla, CA), and all enzymes from Boehringer-Mannheim UK Ltd (Lewes, East Sussex, UK). All other reagents were obtained as previously described.[10,12] The mini plasmid kit Qiavac was obtained from Qiagen Ltd (Crawley, West Sussex, UK), DH5α competent cells from Gibco BRL (Life Technologies, Paisley, UK), a Becton-Dickinson fluorescence-activated cell sorter (FACS III flow cytometer; Cowley, Oxford, UK) was used. PCR products were purified using talent cleanmix from VHBio Ltd, Newcastle, UK. Antiserum raised to the surface protein 5′-nucleotidase of rat neutrophils[13] has been used previously. The pool of anti-human C9 monoclonal antibodies included MC47, 11.60 and 9.48[10,14] and 12.61 were produced by Mrs C. Rogers, UWCM, Cardiff UK.

Computer analysis of sequence similarity

The Wisconsin GCG package was used for database searching for sequence motifs that matched patterns defined in the Prosite dictionary of protein sites and patterns (motifs), regions of similarity to other proteins (bestfit), and the presence of consensus sequences in C9 (findpatterns) as well as general investigations of hydrophobicity, charge distribution and secondary structure predictions (pepplot and plotstructure).

Oligonucleotides

Oligonucleotides not used previously[10] were prepared using an Applied Biosystems 392 DNA synthesizer as follows: C9(25): CTTGTTTTTA TGGTCGTGTA CATTTCTTAC ATCTATGCGA GTTATGACCC AGAG. C9(26): CTTGTTTTTA TGGTCGTGTA CATTTCTTAC ATCTATGCGG CTAACAGAA AGCAG. C9(27): CTTGTTTTTA TGGTCGTGTA CATTTCTTAC ATCTATGCGA GCAGTGGCT CTGCATC. C9(35): CTTGTTTTTA TGGTCGTGTA CATTTCTTAC ATCTATGCGG ACCGCAGAA TGAGCCCCT. C9(36): CTTGTTTTTA TGGTCGTGTA CATTTCTTAC ATCTATGCGG CATCACACAT AGACCGCA. C9(28): CAGAATGAG CCCC**GCTAGT** **GCCGCC**TCAC AATGCGATCC. C9(29): GATCGCATTG TGA**GGCGGCA** **CTAGC**GGGGC TCATTCTG. C9(32): 3' anti-sense removing stop, attaching factor Xa site (bold), hexahistidine tag (underlined), stop and xbaI site (bold), CCCATC**TCTA** **GATTAGTGAT** GGTGATGGTG ATG**CCGGCCC** **TCAATTTTTT** CATTGGGGA. C9(34): 5′ sense attaching BglII site (bold) and rest of honeybee melittin signal peptide (underlined), CCACAC**AGAT** **CTGCGAGAATGAA** ATTCTTAGTC AACGTTGCCC TTGTTTTTAT GGTC.

Generation of cDNA coding for C9 mutants

The N-terminal region of C9 was mutated as illustrated in [Table 1]Table 1b. First stage PCR added half the honeybee melittin signal peptide and a hexahistidine tag at the C-terminus (HIS). The following oligonucleotides were used: C9(25) and C9(32) for mutant 5-538, C9(26) and C9(32) for mutant 9-538, C9(27) and C9(32) for mutant 13-538, C9(35) and C9(32) for mutant 17-538, C9(36) and C9(32) for mutant 21-538, C9(18) and C9(32) for mutant 24-538. Second stage PCR used oligonucleotides C9(34) and C9(32) to attach the remainder of the signal peptide. Mutant (24-538) had no histidine tag. Mutant 27ASAAS31 was made using oligonucleotides C9(28) with C9(32), and C9(29) with C9(11) to make two fragments that were joined by second stage PCR, as described above (see Fig. 1).

Expression of recombinant C9 in insect cells

C9 cDNA from plasmid pSV51HC9 was expressed in insect cells using the method already described[10] with two minor modifications. The multiple cloning site of the baculovirus transfer vector, pVL1392 (R & D Systems, Abingdon, Oxford, UK) was used allowing the DNA fragment to be inserted unidirectionally with BglII and XbaI. A hexahistidine tag was added to the C-terminus to aid purification. Greater than 80% of the colonies contained inserts, orientation of which was confirmed by PCR with oligonucleotides BV5 (5′-sense attaching to pVL1392 upstream of insert site[10]) and C9(32). Mutants were sequenced to confirm mutation (ABI 373 automatic DNA sequencer; Perkin-Elmer, Warrington, UK). Viral stock was prepared as previously descibed[10] using *Trichoplusia ni* (5BI-4) cells maintained in protein-free medium, excel 401, pH 6·2, at 27° (Cambridge Bioscience, Cambridge, UK). Recombinant proteins were eluted from a nickel-agarose column (Qiagen) using an imidazole gradient in 50 mM sodium phosphate buffer, pH 7·4 with 300 nM sodium chloride. Samples were concentrated by centrifugation using Millipore ultrafree filtration units, dialysed with Krebs Hepes buffer (KRH: 120 mM NaCl, 25 mM Hepes, 4·8 mM Kcl, 1·2 mM KH_2PO_4, 1·2 mM $MgSO_4$ and 1·3 mM $CaCl_2$) to remove the imidazole and stored in aliquots at −70°C. The hexahistidine tag did not effect biological activity as shown by comparison

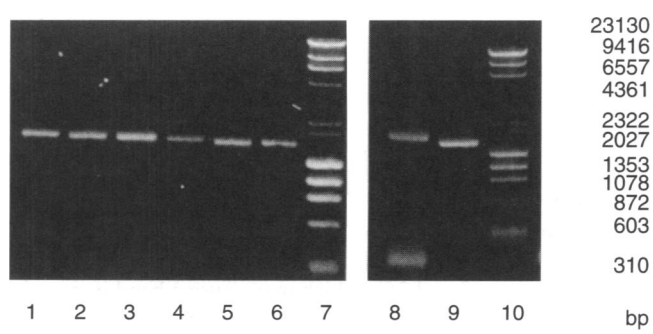

Figure 1 Agarose gel of PCR products of normal and mutated recombinant C9. Agarose gel of final stage PCR products with addition of honeybee mellitin signal peptide and enzyme sites. Lane 1, DNA of mutant (5-538)HIS. Lane 2, DNA of mutant (9-538)HIS, Lane 3, DNA of mutant (13-538)HIS. Lane 4, DNA of mutant (17-538)HIS. Lane 5, DNA of mutant (21-538)HIS, Lane 6, DNA of mutant (24-538), Lanes 7 and 10, DNA standards, Lane 8, DNA of full-length 27ASAAS31, Lane 9, DNA of large fragment of 27ASAAS31.

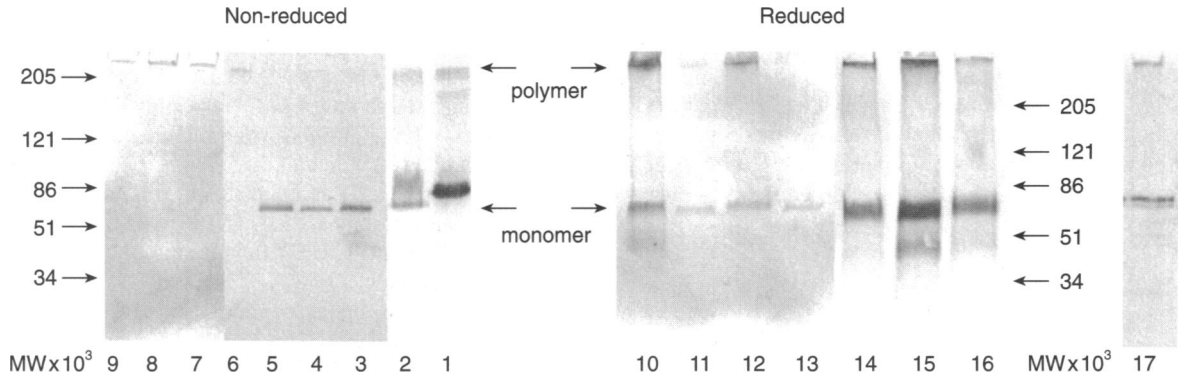

Figure 2. Immunoblot of normal and mutated recombinant C9. Samples (20 μl insect cell supernatant or 100 ng normal human C9, reduced or non-reduced) were subjected to 4–20% gradient SDS–PAGE prior to western blotting onto nitrocellulose, reacting with a pool of C9 monoclonal antibodies (see Materials and Methods), and developing with alkaline phosphatase. Lanes 1–9 are non-reduced and lanes 10–16 are reduced. Lane 1, normal human C9; Lane 2, normal recombinant C9 with HIS tag; Lanes 3 and 13, mutant (5-538)HIS; Lanes 4 and 12, mutant (9-538)HIS; Lanes 5 and 11, mutant (13-538)HIS; Lanes 6 and 10, mutant 27ASAAS31 with HIS tag; Lanes 7 and 14, mutant (17-538)HIS; Lanes 8 and 15, mutant (21-538)HIS; Lanes 9 and 16, mutant (24-538), Lane 17, normal human C9 incubated with zinc for six hours (see ref. 10 for details).

of lysis of pigeon erythrocytes by normal human C9, recombinant C9 with no histidine tag and recombinant C9 with a histidine tag (Fig. 3a).

C9-dependent lysis of pigeon erythrocytes and rat peritoneal neutrophils

Lysis of pigeon erythrocytes was detailed previously.[12] Rat neutrophils (more than 98% pure) were freshly prepared from peritoneal exudate of rats as previously described.[15] The same amount of C9 (500 ng/ml) was added in each experiment, as judged by densitometric analysis of the monomer band produced by immunoblotting each sample in non-reduced conditions. Measurements of mutated recombinant protein were repeated at least three times and compared to normal recombinant C9 in each experiment. In order to test how much recombinant C9 was bound to C5b-8 sites on rat neutrophils, every effort was taken to ensure the same number of sites were present for each test. This was achieved by making C5b-8 sites on rat neutrophils before separating the cells for each different mutant.

RESULTS

Prediction of key domains involved in the polymerization of C9

Computer modelling software that predicted the secondary structure, charge distribution and sequence similarity to other C9s, cytolysins and membrane proteins, was used to identify key domains at the N-terminus of C9 that might prevent self polymerization. The N-terminal region of C9 contains two proline residues (8 and 26), which may cause a bend in protein structure, and although there is a known disulphide bond between C22 and C57,[16] removal of this domain may expose parts of the protein not normally exposed in the globular protein. This was supported by analysis of sequence similarity between different C9s, showing low similarity in the initial 18 residues of C9 (range 18–54%) compared to high similarity in residues 19–47 (range 62–93%) (Table 1a). We have located a cytokine receptor family signature motif in human C9 (27WSEWS31) (Table 2a), which was present in every C9 so

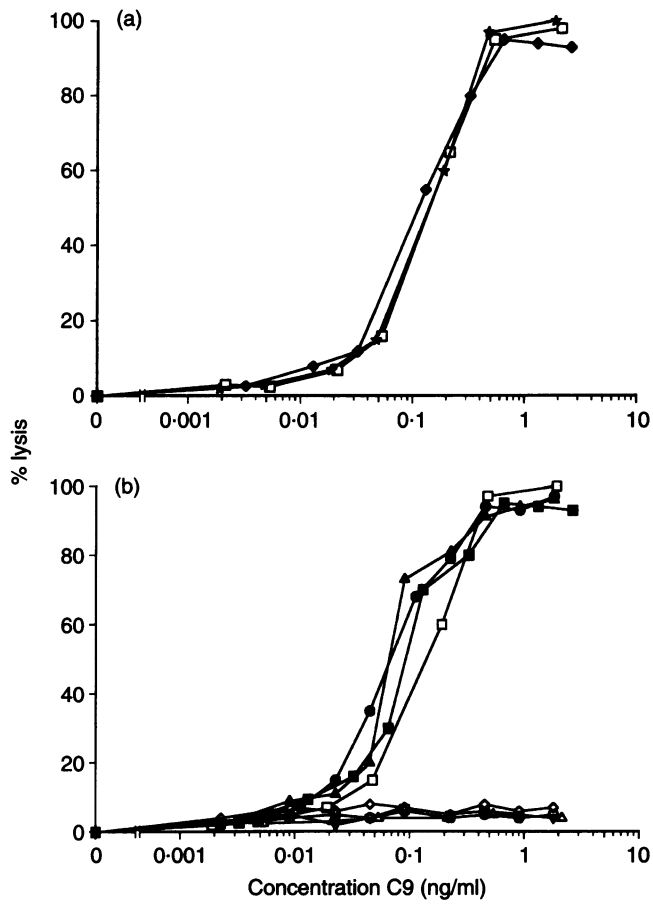

Figure 3. Haemolytic activity of normal and recombinant C9 on erythrocytes. (a) Effect of C-terminal polyhistidine tag on haemolytic activity of normal and recombinant C9 on erythrocytes. (b) Effect of N-terminal deletion of amino acids on haemolytic activity of normal and recombinant C9 on erythrocytes. ★ normal C9; ◆ recombinant C9 without HIS tag; □ recombinant C9 with HIS tag; ■ mutant (5-538)HIS; ● mutant (9-538)HIS; ▽ mutant (13-538)HIS; ○ mutant (17-538)HIS; ▲ mutant (21-538)HIS; ◇ mutant (24-538); ▽; 27ASAAS31 with HIS tag.

Table 1. The N-terminal sequuence of different C9's and list of all C9 mutations (**A**) Amino acids 1–47 at the N terminus of C9 (similar regions (5/6) in bold). Key sequences are underlined and detailed in the text. Residues 1–6 fit a consensus calcium binding site motif, residues 27–31 fit a cytokine receptor motif, and residues 42–47 fit a consensus serine phosphorylation site (**B**) List of six human C9 N-terminal deletion mutations expressed in insect cells and mutant altering 27WSEWS31 to 27ASAAS31

(a)

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Residues    = 1                        24                        47
              |                        |                         |
Human C9    = QYTTSYDPELTESSGSASHIDCRMSPWSEWSQCDPCLRQMFRSRSIE
Horse C9    = GPTPNYAPEPEQQSGTPLPIDCRMSSWSEWSECDPCLRQMFRSRSIE
Mouse C9    = QMPIPVSREEQEQH.YPIPIDCRMSPWSNWSECDPCLKQRFRSRSIL
Rabbit C9   = GPTPSYVHEPIQRSDPLQPIDCRMSPWSEWSHCDPCLRQMFRSRSIE
Rat C9      = QAPEPTPREEPSADALL PIDCRMSTWSQWSQCDPCLKQRFRSRSME
Trout C9    = QANGTLGRSRWL ... PLDPVDCVWSRWSEWTPCNSCTKIRHRSRSVE
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(b)

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(5–538aa)   =      SYDPELTESSGSASHIDCRMSPWSEWSQCDPCLRQMFRSRSIE
(9–538aa)   =           ELTESSGSASHIDCRMSPWSEWSQCDPCLRQMFRSRSIE
(13–538aa)  =                SSGSASHIDCRMSPWSEWSQCDPCLRQMFRSRSIE
(17–538aa)  =                     ASHIDCRMSPWSEWSQCDPCLRQMFRSRSIE
(21–538aa)  =                          DCRMSPWSEWSQCDPCLRQMFRSRSIE
(24–538aa)  =                             MSPWSEWSQCDPCLRQMFRSRSIE
27ASAAS31 = QYTTSYDPELTESSGSASHIDCRMSPASAASQCDPCLRQMFRSRSIE
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far sequenced: i.e. human, mouse, rabbit, rat, trout and horse C9, as well as C6, C7, C8α C8β, T-cell perforin and properdin. A similar tryptophan-rich sequence (WEWWR) was found in pore-forming thiol-activated cytolysins, five residues extracellularly from the cholesterol binding site (Table 2b).

Using a consensus sequence for calcium binding proteins,[17] a putative Ca^{2+} binding loop was found at the N-terminus of C9, residues 1–16 (Table 1a), containing a strong negative charge, making it a good candidate for a cation binding site. The predicted site was rich in oxygen containing amino acid side chains, similar to domains in EF-hand Ca^{2+} binding proteins where evolution has selected oxygen rather than nitrogen for 8 co-ordination of Ca^{2+} to be selective over Mg^{2+}.[17] A potential serine phosphorylation site 42RXXSXE47 was also identified, consistent with the report that C9 can be serine phosphorylated near the N-terminus.[18]

These results highlighted the need to remove or mutate amino acids within the N-terminus of C9, and test whether this region had a role in protecting globular C9 from self-polymerization. The mutants generated (Table 1b) were expressed in insect cells using baculovirus infection (Table 3).

The effect of N-terminal mutation on the self-polymerization of C9

It is well established that only correctly folded proteins are able to enter the secretory pathway of cells.[19,20] All the N-terminal mutants presented here were secreted from insect cells into the supernatant, implying that they were correctly folded. We have evidence that mis-folded C9 is degraded within the cell and not secreted.[12] The C9 mutants were compared on gel electrophoresis under reducing and non-reducing conditions and in their ability to bind a pool of monoclonal antibodies to native C9 (Fig. 2) to gauge the production of polymer C9. Removal of 4, 8 or 12 amino acids from the N-terminus did not effect polymerization as judged by immunoblotting the insect cell supernatant in non-reduced conditions (Fig. 2, lanes 1–5). In contrast, removal of 16, 20 or 23 amino acids produced proteins containing no free monomeric C9 under non-reducing conditions (Fig. 2, lanes 7–9) and a band of high molecular mass, that did not react well with antibodies, indicating formation of aggregates or polymer. However, under reducing conditions, a band of high molecular mass material was still evident, indicating the presence of strong covalently linked full-ring polymer that was resistant to reduction and SDS (Fig. 2, lanes 14–16). This can be compared to lane 17 showing normal human C9 full-ring polymer after incubation with zinc at 37° for 6 hr.[10] A monomer band was also visible, indicating the presence of aggregates or non-ring polymer which only disappeared completely after incubation at 37° for 3 days.[10] This suggested a proportion of mutant polymer was not as tightly covalently linked as the normal ring polymer in the MAC, which is non-dissociable. Mutant 27ASAAS31 also self-polymerized (Fig. 2, lanes 6 and 10), suggesting that the WSEWS motif was important for maintaining the correct folding of the N-terminus and preventing it from opening out prematurely.

Haemolytic activity of recombinant C9 mutants on pigeon erythrocytes

The haemolytic activity of recombinant mutants on pigeon erythrocytes fell into two categories, those with normal or enhanced haemolytic activity (mutants 5-538, 9-538, and 13-538) and those without (mutants 17-538, 21-538, 24-538 and 27ASAAS31) (Fig. 3b). These differences in haemolytic activity reflected the presence (mutants 5-538, 9-538 and 13-538) or absence (mutants 17-538, 21-538 24-538, and 27ASAAS31) of monomer C9 in non-reduced conditions (shown in Fig. 2) from the mutated or normal recombinant C9 purified from the insect cell medium.

These results suggest that removal of 16 or more amino

Table 2. Proteins from Genbank database containing WSXWS (A) or WEWWR (B) motifs (a)

Protein	Accession number	Motif	Start AA	Total AA	Sequence
Human C9	P02748	WSXWS	27	538	RMSPWSEWSQCDP
Horse C9	U19381	WSXWS	27	526	RMSSWSEWSECDP
Mouse C9	P06683	WSXWS	26	528	RMSPWSNWSECDP
Rabbit C9	U20055	WSXWS	27		RMSPWSEWSHCDP
Rat C9	U52948	WSXWS	26	534	RMSTWSQWSQCDP
Trout C9	P06682	WSXWT	24		VWSRWSEWTPCNS
Human C6	P13671	WSXWS	571	934	QWGCWSSWSTCDA
Human C7	P10643	WSXWS	503,506	843	DGGWSCWSSWSPCV
Human C8α	P07357	WSXWS	542,545	584	GSWSCWSSWSVCR
Human C8β	p07358	WSXWS	551	591	KWNCWSNWSSCSG
Perforin	x13224	WSXWG	425	556	FIQAWSLWGDWFT
Properdin	p27918	WSXWS	80	469	RSPRWSLWSTWAP
Properdin	p27918	WSXWS	382	469	LKGSWSEWSTWG
Cytokine rec β-chain	p32927	WSXWS	425	897	GIWSEWSEAR
Human CNTFR	P26992	WSXWS	290	372	EIGTWSDWSVAA
Human EPOR	P19235	WSXWS	233	508	FGGFWSAWSEPVS
Human G-CSFR	Q99062	WSXWS	318	836	LPGHWSDWSPSL
Human GM-CSFR	P15509	WSXWS	306	400	RILNWSSWSEAI
IL2R-beta	P14784	WSXWS	220	551	EFTTWSPWSQPLA
IL4-R alpha	P24394	WSXWS	212	825	YNTTWSEWSPST
IL5-R alpha	Q01344	WSXWS	322	420	EAGLWSEWSQPI
IL6-R alpha	P08887	WSXWS	300	468	GQGEWSEWSPEA
IL6-R beta	P40189	WSXWS	310	918	GKGYWSDWSEEA
IL7-R alpha	P16871	WSXWS	217	459	FKGFWSEWSPSY
IL9-R	Q01113	WSXWS	245	522	YTGQWSEWSQPV
Prolactin-R	P16471	WSXWS	215	622	DHGYWSAWSPAT
ThrombopoeitinR	P40238	WSXWS	474	635	YQGPWSSWSDP
Thrombospondin 1	P07996	WSXWS	438,441	1170	DGGWSHWSPWSSC
Thrombospondin 2	P35442	WSXWS	440,443	1172	DGGWSHWSPWSSC
Thrombospondin 2	P35442	WSXWS	497,500	1172	DGRWSPWSPWSAC
Colicin B	p17998	WSXWS	83	697	EEGDWSGWSVSV
Colicin D	p05819	WSXWS	82	510	EEGDWSGWSVSV
(b)					
Alveolysin	P23564	WEWWR	466	501	CTGLAWEWWRTVVD
Perfringolysin-O	P19995	WEWWR	464	500	CTGLAWEWWRDVIS
Pneumolysin	P11990	WEWWR	432	470	CTGLAWEWWRTVYE
Streptolysin-O	P21131	WEWWR	535	571	CTGLAWEWWRKVID
Listeriolysin-O	P13128	WEWWR	489	529	CTGLAWEWWRTVID
Ivanolysin	P31831	WEWWR	488	528	CTGLAWEWWRTVVD
Seeligeri	P31830	WEWWR	490	530	CTGLFWEWWRTVID

acids exposes a region of C9 that allows polymerization to occur without C5b-8 and inactivates the protein.

Effect of mutants on neutrophil permeability and vesiculation

Neutrophils and other nucleated cells exhibit a series of permeabilty thresholds following C9 attack. First, a rapid influx of Ca^{2+}, followed by permeability to propidium iodide (PI), and then lysis, or removal of the MAC by vesiculation.[3,21] The effect of mutation at the N-terminus on PI permeability was investigated using FACS analysis. Our results have confirmed the sequence of threshold events previously illustrated by this method[21] and show how the timing of this sequence can change by mutating the N-terminus of C9.

Cells attacked by normal C9 did not become PI positive before increasing in size, as judged by an increase in forward scatter at 6 min, returning to normal within 15 min. These cells then either recovered, remaining negative to PI, or became positive to PI followed by lysis or recovery. Table 3 shows the percentage of cells that were positive to propidium iodide after 15 min of incubation with C9 under the same conditions as those used for Fig. 4. The end point lysis was measured by incubating the cells with C9 for 60 min before adding propidium iodide. Two hyperactive mutants (5-538 and 9-538) caused the cells to react sooner (less than 3 min) than normal recombinant C9, and all three mutants maintained the effect for longer than the normal recombinant C9, taking 30 min to return to normal (Fig. 4a). This was not due to a concentration effect of C9 since four times the dose of normal recombinant C9 (2 μg/ml) produced the same effect as 500 ng/ml (results not shown). After 15 min of complement attack a population of cells emerged that were small in size (very low forward scatter)

Table 3. Expression of normal and mutant recombinant C9 in insect cells

Recombinant C9	Non-reduced C9 (μg/ml)*	% Cells PI positive†	End point lysis‡	Vesicle production§
Normal human C9		21·4±1·7	13·9±1·9	9·9±2·4
Recombinant C9 no HIS	4·7±0·4			
Recombinant C9-HIS	5·8±0·3	24·3±2·1	14·2±2·1	10·6±4·6
Mutant (5–538aa)HIS	5·9±0·4	22·3±1·5	17·5±2·3	16·2±4·9
Mutant (9–538aa)HIS	5·0±0·3	23·4±1·9	19·3±0·6	21·5±7·8
Mutant (13–538aa)HIS	4·7±0·5	25·1±3·4	21·7±0·6	18·8±7·4
Mutant (27ASAAS31)HIS	0·7±0·3			
Mutant (17–538aa)HIS	0·6±0·4			
Mutant (21–538aa)HIS	0·4±0·3			
Mutant (24–538aa)	0·3±0·2			

*Concentration of recombinant C9in insect supernatant as judged by monomer band after non-reducing SDS–PAGE and immunoblot.

†Percentage of total cells positive to PI after 15 min of incubation with C9 under the same conditions as Fig. 4. The PI was added at the time of C9 addition.

‡Percentage lysis of cells. Percentage of cells positive to PI when added after incubation with C9 for 60 min.

§Percentage of total cells in population not fluorescent to PI and of small size after incubation with C9 for 45 min.

and not fluorescent to PI. The appearance of this population after 15 min was consistent with neutrophil recovery by vesicle formation. The three mutants tested had more cells in this population after incubation for 45 min than the normal recombinant C9 (Table 3, vesicle production). We have not collected this population for further study, and therefore cannot rule out the possibility that they were contaminated by fragments of dead cells, but suggest they are vesicles due to their size, lack of fluorescence to PI and time of production. The physical size of this population was the same (as judged by forward scatter) for normal and the mutant recombinant C9.

Effect of deletion of up to 12 N-terminal residues on the binding of C9 to neutrophils

To investigate whether the enhanced activity of mutants 5-538, 9-538, and 13-538 was the result of increased binding to C5b-8, FACS analysis was used to quantify C9 antibody binding of various C9s to C5b-8 sites on rat neutrophils. All three mutants bound 50% more to C5b-8 sites at 4° than normal recombinant C9 (Fig. 4b), which remained elevated on warming to 37°, confirming that their increased activity was due to increased binding to C5b-8 sites on cells.

DISCUSSION

The results reported here have important implications for the mode of action of C9, confirming our hypothesis that the N-terminus of C9 is crucial for protection of the globular protein against polymerization and irreversible inactivation. Removal of 12 amino acids from the N-terminus of C9 did not reduce biological activity (Fig. 3b), whereas removal of 16 or more inactivated the protein by self-polymerization (Figs 2 and 3b). This result agrees with our computer-aided hypothesis that the N-terminus of C9 is bent, hiding a region critical for polymerization.

The 12 N-terminal amino acids of C9 exhibit a strong negative charge and the 16 N-terminal amino acids contain a consensus sequence[17] for calcium binding proteins (Table 1a). Clearly these residues are necessary for the predicted protection by the N-terminus, as removal (mutant 17-538) resulted in a polymerized protein. Partial removal of this region (mutants 5-538, 9-538, and 13-538) resulted in C9 with a two–threefold greater haemolytic activity on erythrocytes (Fig. 3b) and 50% increased binding to C5b-8 sites on neutrophils (Fig. 4b). The observed result that an increased dose of normal C9, and presumably increased binding to cells, did not produce the same result as the mutants by FACS analysis suggests that the effect of the mutants was not just one of increased binding to cells. The formation of the mutants in the MAC may have been different. The results are however, consistent with removal of a few residues causing premature unfolding of the N-terminus enabling the partially unfolded protein to bind cells more readily.

C9 belongs to a heterogeneous group of proteins, including thiol-activated cytolysins,[22] T-cell perforin,[23] many bacterial toxins,[24,25] bee melittin,[26] some viral proteins, and several invertebrate toxins,[27] which insert themselves into the plasma membrane of cells. This is followed by aggregation, and sometimes cross-linking, which in the case of complement component C9 involves polymerization through disulphide cross-bridges. We have identified a motif in C9, 27WSEWS31, that is common to a family of cytokine receptors (Table 2a), where it resides extracellularly a few residues from the membrane. A similar tryptophan-rich motif (WEWWR) in the pore-forming thiol-activated cytolysins (Table 2b) is also situated extracellularly five residues from the cholesterol binding cysteine.[22] Cytolysins produce pores by binding membrane cholesterol and oligomerizing. The membrane spanning domain of C9 is located between residues 293–334,[1] clearly not adjacent to this motif unless it interacts with it. The C9 motif (27WSEWS31) is preceded by a cysteine residue at position 22, the same relative position upstream as in the cytolysin motif. There is also a proline residue in C9 at position 26 producing a predicted angle of 10·4 nm compared to 0·1 nm

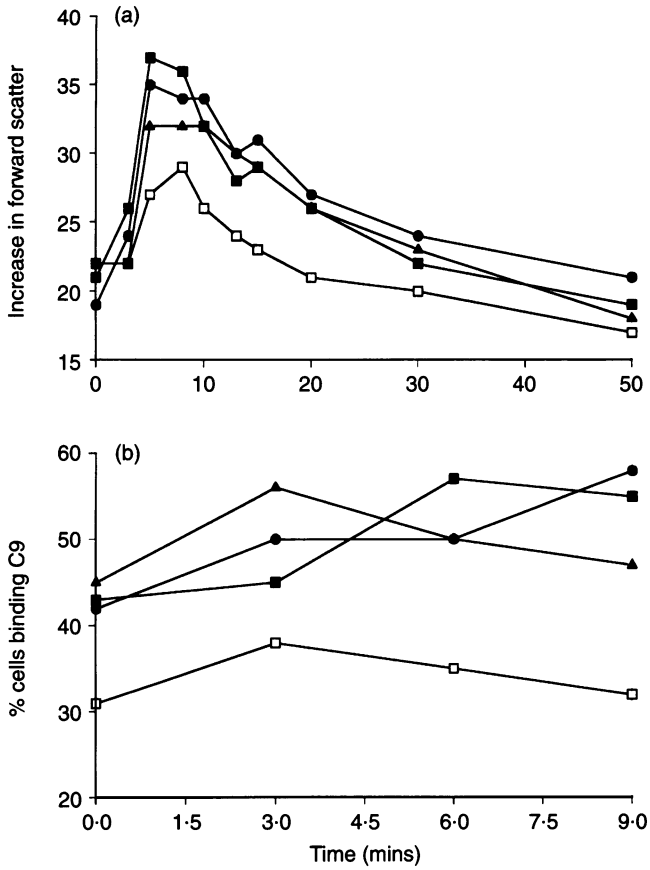

Figure 4. FACS analysis of the effect of normal and recombinant C9 on rat neutrophils. (a) Graph showing increasing forward scatter with time after C9 addition to C5b-8 sites on rat neutrophils. C5b-8 sites were prepared on rat neutrophils (10^6/ml) by incubating with antiserum (1/20) on ice for 30 min. The cells were then resuspended in fresh KRHA and incubated with C9-depleted human serum (1/10) at 37° for 30 min before separating into different tubes for C9 addition. Propidium iodide (1 μg/ml in KRHA), used to investigate the permeability thresholds induced by complement attack, was added at the time of C9 (500 ng/ml) addition and the change in fluorescence was measured with time by FACS analysis. (b) Binding of C9 to C5b-8 sites on rat neutrophils after 30 min on ice (time 0) followed by warming to 37°. To investigate the binding of C9 (500 ng/ml) to C5b-8 sites on rat neutrophils, cells were incubated with C9 at 0° for 30 min followed by incubation at 37° for 0–9 min. The cells were then incubated at 0° for 30 min each with a C9 antibody that only recognized C9 in the MAC (C9-B7, kindly provided by Catherine Rogers, UWCM) and a fluorescein isothiocyanate (FITC)-conjugated second antibody prior to FACS analysis. Deletion of 4, 8, or 12 amino acids from the N-terminus of C9 increased the ability of the protein to bind C5b-8 sites on rat neutrophils. □ recombinant C9 with HIS tag; ■ mutant (5-538)HIS; ● mutant (9-538)HIS; ▽ mutant (13-538)HIS.

for the cytolysin motif, when analysed by computer modelling software. This suggests a bend in the motif which may be important for preventing its premature exposure. The motif WSEWS has been shown to have a role in the activity of other transmembrane proteins. For example, mutation of WSEWS in the human granulocyte–macrophage colony-stimulating factor (GM-CSF) receptor destroyed activity,[28] mutation of the motif in the growth hormone receptor disrupted ligand binding and receptor signalling,[29] and this motif may contribute to efficient receptor folding in the erythropoietin receptor.[30] This motif is obviously important for the activity of C9, as mutant 27ASAAS31 produced an inactive self-polymerized protein. The question arises whether it is involved with the membrane insertion of C9 or the interaction with other regions to prevent premature polymerization.

The protecting effect of the N-terminal domain demonstrated here has been found in other proteins unrelated to pore formers. For example, we have proposed that some bioluminescent proteins create a solvent cage required for high quantum yield photon emission by folding of the C- or N-terminus.[31] Removal of the C-terminal 12 amino acids of beetle luciferases destroys activity, which can be restored by peptides of sequences unrelated to the original protein. The question now arises whether this can be achieved with C9. Furthermore, whilst there is a high sequence similarity (60–80%) between the beetle luciferases from different species, the sequence similarity is much less in the C-terminal domain. Similarly, in the C9s so far sequenced there is little or no sequence similarity in the first 18 residues of human C9 (range 18–54%) compared to high sequence similarity in residues 19–47 (range 45–95%) (Table 1a). Our results and computer analysis of C9 are consistent with the concept that peptides that have evolved to protect other domains in proteins do not need a conserved primary sequence. What matters is the polarity, enabling the protecting peptide to bend over and bind to other sites in the protein. The stimulus that breaks this interaction when C9 binds to C5b-8 in the cell membrane will expose the new active site and start the molecular sequence of events for C9 attack.[3] It will be interesting to investigate whether the potential serine phosporylation site at 42–47 is necessary for N-terminal interaction.

In conclusion, the results reported here shed new light on how globular C9 inserts and polymerizes in the plasma membrane. The question now arises how the unfolding at the N-terminus, predicted by our results, relates to the signal for removal of the MAC, and whether activation of the protection mechanism requires polymerization of C9 to occur. Our data indicate a new approach to engineering C9 which could lead to therapeutic procedures enabling the effects of pore-forming proteins to be controlled *in vivo* in inflammatory disease.

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REFERENCES

1. Stanley K.K., Kocher H.P., Luzio J.P., Jackson P. & Tschopp J. (1985) The sequence and topology of human complement component C9. *EMBO J* **4**, 375.
2. Stanley K.K., Page M., Campbell A.K. & Luzio J.P. (1986) A mechanism for the insertion of complement component C9 into target membranes. *J Immunol* **23**, 451.
3. Campbell A.K. (1994) *Rubicon: The Fifth Dimension of Biology*. Duckworth and Co Ltd, London.
4. Dankert J.R. & Esser A.F. (1985) Proteolytic modification of

human complement protein C9: loss of poly (C9) and circular lesion formation without impairment of function. *Proc Natl Acad Sci USA* **82,** 2128.

5. TSCHOPP. (1984) Circular polymerisation of the membranolytic ninth component of complement. Dependence on metal ions. *J Biol Chem* **259,** 10 569.
6. YAMAMOTO K. & MIGITA S. (1981) Mechanisms for the spontaneous formation of covalently linked polymers of the terminal membranolytic complement protein (C9). *J Biol Chem* **258,** 7887.
7. TSCHOPP J., MULLER-EBERHARD M.J. & PODACK E.R. (1982) Formation of transmembrane tubules by spontaneous polymerization of the hydrophilic complement protein C9. *Nature* **298,** 534.
8. CAMPBELL A.K. & MORGAN B.P. (1985) Monoclonal antibodies demonstrate protection of polymorphonuclear lymphocytes against complement attack. *Nature* **317,** 164.
9. STEIN J.M. & LUZIO J.P. (1991) Ectocytosis caused by sublytic autologous complement attack on human neutrophils. *Biochem J* **274,** 381.
10. TAYLOR K.M., MORGAN B.P. & CAMPBELL A.K. (1994) Altered glycosylation and selected mutation in recombinant human complement component C9: effects on haemolytic activity. *Immunology* **83,** 501.
11. LAINE R.O. & ESSER A.F. (1989) Detection of refolding conformers of complement protein C9 during insertion into membranes. *Nature* **341,** 63.
12. TAYLOR K.M., LUZIO J.P. & CAMPBELL A.K. (1994) A method for *in vitro* synthesis of unglycosylated recombinant complement component C9. *J Immunol Methods* **167,** 129.
13. CAMPBELL A.K., DAW R.A., HALLETT M.B. & LUZIO J.P. (1981) Direct measurement of the increase in intracellular free calcium ion concentration in response to the action of complement. *Biochem J* **194,** 551.
14. CAMPBELL A.K. & MORGAN B.P. (1985) Monoclonal antibodies demonstrate protection of polymorphonuclear leukocytes against complement attack. *Nature,* **317** 164.
15. HALLETT M.B., LUZIO J.P. & CAMPBELL A.K. (1981) Stimulation of calcium-dependent chemiluminescence in rat polymorphonuclear leukocytes by polystyrene beads and the nonlytic action of complement. *Immunology* **44,** 569.
16. LENGWEILER S., SCHALLER J. & RICKLI E.E. (1996) Identification of disulphide bonds in the ninth component (C9) of human complement. *FEBS Letters* **380,** 8.
17. PERSECHINI A., MONCRIEF N.D. & KRETSINGER R.H. (1989) The EF-hand family of calcium modulated proteins. *TINS* **12,** 462.
18. PAAS Y. & FISCHELSON Z. (1995) Shedding of tyrosine and serine/threonine ecto-protein kinases from human leukemic cells. *Arch Biochem Biophys* **316,** 780.
19. HURTLEY S.M. & HELENIUS A. (1989) Protein oligomerisation in the endoplasmic reticulum. *Annu Rev Cell Biol* **5,** 277.
20. PELHAM H.R.V. (1989) Control of protein exit from the endoplasmic reticulum. *Annu Rev Cell Biol* **5,** 1.
21. PATEL A.K. & CAMPBELL A.K. (1987) The membrane attack complex of complement induces permeability changes via thresholds in individual cells. *Immunology* **60** 135.
22. IWAMOTO M., NAKAMURA M., MITSUI K., ANDO S. & OHNO-IWASHITA Y. (1993) Membrane disorganisation induced by perfringolysin O (theta-toxin) of *Clostridium perfringens*-effect of toxin binding and self-assembly on liposomes. *Biochim Biophys Acta* **1153,** 89.
23. PEITSCH M.C., AMIGUET P., GUY R., BRUNNER J., MAIZEL J.V. & TSCHOPP J. (1990) Localisation and molecular modelling of the membrane-inserted domain of the ninth component of human complement and perforin. *Mol Immunol* **27,** 589.
24. BHAKDI S. & TRANUM-JENSEN J. (1991) Alpha-toxin of *Staphylococcus aureus. Microbiol Rev* **55,** 733.
25. VAN DER GOOT F.G., PATTUS F., WONG K.R. & BUCKLEY J.T. (1993) Oligomerisation of the channel-forming toxin aerolysin precedes insertion into lipid bilayers. *Biochemistry* **32,** 2636.
26. LAINE R.O., MORGAN B.P. & ESSER A.F. (1988) Comparison between complement and melittin hemolysis: anti-melittin antibodies inhibit complement lysis. *Biochemistry* **27,** 5308.
27. BHAKDI S. & TRANUM-JENSEN J. (1987) Damage to mammalian cells by proteins that form transmembrane pores. *Rev Physiol Biochem Pharmacol* **107,** 147.
28. RONCO I.V., DOYLE S.E., RAINES M., PARK L.S. & GASSON J.C. (1995) Conserved amino acids in the human granulocyte–macrophage colony-stimulating factor receptor-binding subunit are essential for tyrosine phosphorylation and proliferation. *J Immunol* **154,** 3444.
29. BAUMGARTNER J.M., WELLS C.A., CHEN C.M. & WATERS M.J. (1994) The role of the WSXWS equivalent motif in growth hormone receptor function. *J Biol Chem* **269,** 29 094.
30. HILTON D.J., WATOWICH S.S., KATZ L. & LODISH H.F. (1996) Saturation mutagenesis of the WSXWS motif of the erthyropoietin receptor. *J Biol Chem* **271,** 4699.
31. WAUD J.P., SALA-NEWBY G.B., MATTHEWS S.B. & CAMPBELL A.K. (1996) Engineering the C-terminus of firefly luciferase as an indication of covalent modification of proteins. *Biochim Biophys Acta* **1292,** 89.