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Altered glycosylation and selected mutation in recombinant human complement component C9: effects on haemolytic activity

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SUMMARY

Recombinant wild-type and mutated forms of human complement component C9 have been synthesized in baculovirus-infected insect cells. Wild-type recombinant C9 was indistinguishable from native C9, as judged by haemolytic activity, trypsin and α-thrombin digestion, reaction with antibodies to C9, enzymatic deglycosylation to the same core size and polymerization in the presence of Zn^{2+}. Replacement of the native signal peptide with the honey-bee melittin signal peptide, and replacement of *Spodoptera frugiperda* (Sf9) cells with *Trichoplusia ni* cells produced yields of 5 μg C9/ml supernatant. Three C9 mutants were generated; one mutant, with four acidic residues changed to alanines in a putative calcium-binding site, had the same biological activity as recombinant C9. Another mutant, lacking 23 N-terminal amino acids, previously showing increased polymerization when produced *in vitro*, polymerized on secretion, rendering it inactive. It was not possible to demonstrate haemolytic activity of the third mutant, cysteines 33 and 36 mutated to alanine, as it was secreted a hundredfold less than the wild-type protein.

INTRODUCTION

Human complement component C9, a glycoprotein of 538 amino acids and a molecular weight (MW) on SDS–PAGE of 71 000[1] is the terminal component of the complement pathway and is required for the rapid 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. Nucleated cells can remove this membrane attack complex (MAC) by vesiculation[4,5] allowing recovery. The polymerization of C9 is thought to play a key role in this sequence of events.[6]

We have produced biologically active unglycosylated C9, in a cell-free system using the polymerase chain reaction (PCR) coupled with *in vitro* transcription and translation.[7] This C9 polymerized more readily than native C9 and produced insufficient monomer for full functional analysis. The generation of large quantities of glycosylated protein was required to allow characterization. The secretion of 0·7 μg/ml recombinant C9 with full haemolytic activity has been reported in insect cells.[8] However, the secretion of this C9 was not optimized and the glycosylation state, which we have shown to affect polymerization,[7] was not reported.

The aim of this study was threefold: to optimize the production of recombinant C9 using different signal peptides and insect cells; to characterize the recombinant C9 in terms of the extent of glycosylation, reaction with antibodies against native C9, its enzyme cleavage pattern, and polymerization in the presence of Zn^{2+}; and to generate mutants with altered polymerization potential. We report here how mutation by PCR and altered glycosylation, by production in insect cells, effects the haemolytic activity of recombinant complement component C9.

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MATERIALS AND METHODS

Materials

Magic PCR-prep was obtained from Promega (Southampton, UK), Geneclean from Bio 101 Inc. (La Jolla, CA), and all enzymes from Boehringer Mannheim (Lewes, UK). All other reagents were obtained as previously described.[7] The anti-human C9 monoclonal antibodies MC34, MC47, MC8, 11.60 and 9.48 and a polyclonal antiserum were prepared by standard procedures.[9] The antibodies 11.60 and 9.48 have not been reported previously and were produced by Mrs C. Rogers (UWCM, Cardiff, UK).

Oligonucleotides

Oligonucleotides, prepared using an Applied Biosystems 381A DNA synthesizer (Warrington, UK), were as follows. C9(5): 3′ anti-sense attaching a *Sal*I site (underlined), CCCCGTCGA CCCAACAGCT CTATTTTTCA TT; C9(7): 5′ sense for mutating cysteines 33 and 36 (underlined) CAAGCCGATC CTGCCCTCAG ACAAATG; C9(8): 3′ anti-sense for mutating cysteines 33 and 36 (underlined) TCTGAGGGCA GGATCGGCTT GTGACCATTC; C9(9): 5′ sense for C9 with native signal peptide (bold) and *Bgl*II site (underlined),

CCACACAGAT CTCAGCATGT CAGCCTGCCGG; C9(11): 5′ sense attaching half of the honey-bee melittin signal peptide (underlined), CTTGTTTTTA TGGTCGTGTA CATTTCTTAC ATCTATGCGC AGTACACGAC CAGT; C9(12): 5′ sense attaching *Bgl*II site (bold) and rest of the honey-bee melittin signal peptide (underlined), CCACACAGAT CTAGAATGAA ATTCTTAGTC AACGTTGCCC TTGTTTTTAT GGTC; C9(13): 3′ anti-sense and *Bgl*II site (underlined), CCCATCAGAT CTCTATTTTT CATTGGGGA; C9(16): 3′ anti-sense mutating residues 109–112 to alanine (underlined), CTCACTTTCA CATGCGGCTG CGGCTGAAAA GTC; C9(17): 5′ sense mutating residues 109–112 to alanine (underlined), GCCGCAGCCG CATGTGAAAG TGAG; C9(18): 5′ sense attaching half of the honey-bee melittin signal peptide (underlined) and removing 23 N-terminal amino acids, CTTGTTTTTA TGGTCGTGTA CATTTCTTAC ATCTATGCGA TGAGCCCCTG GAGT; BV3: 3′ anti-sense to the downstream region of pVL941, CAACAACGCA CAGAATCTAG; BV5: 5′ sense to the upstream region of pVL941, TTTACTGTTT TCGTAACAGT TTTG.

Expression of recombinant C9 in insect cells

The C9 cDNA was removed from plasmid pSV51HC9 using a PCR procedure already described.[7] The PCR products were inserted into the baculovirus transfer vector, pVL941 (R&D Systems, Abingdon, UK), at the *Bam*HI cloning site. This vector contained the *Autographa californica* mononuclear polyhedrosis virus (AcMNPV) polyhedrin gene promoter and recombination sequences. A C9 construct containing the native human signal peptide was prepared by PCR with oligonucleotides C9(9) and C9(13), and another containing the honey-bee melittin signal peptide was produced using two-stage PCR with oligonucleotides C9(11) and C9(5), followed by C9(12) and C9(13) (Fig. 2). PCR products were treated with Magic PCR-prep to remove oligonucleotides, and cDNA was purified by phenol-chloroform extraction and ethanol precipitation. Protein and salts were removed using a Geneclean kit. Constructs (1 μg/100 μl) were digested with *Bgl*II, and pVL941 plasmid (3 μg/100 μl) was digested with *Bam*HI (16 U/100 μl) and treated with calf intestinal alkaline phosphatase (9 U/100 μl).[10] C9 inserts were subcloned into *Escherichia coli* HB101 using calcium chloride and heat shock.[10] Colonies growing on ampicillin plates were screened for cDNA inserts by PCR using oligonucleotides BV5 and BV3. Approximately 2–10% of the colonies contained inserts, the orientation of which was confirmed by PCR. Insect cells were co-transfected with a linearized AcMNPV DNA containing a lethal deletion in the polyhedrin promoter region, enabling higher rates of recombination (Baculogold kit from AMS Biotechnology, Whitney, UK). Plasmids from three different bacterial clones were co-transfected for each recombinant C9 to ensure no random mutations were produced during PCR, and eliminating the need to sequence mutants.

Culture supernatant containing recombinant virus was harvested 5 days after co-transfection. Virus was isolated by plaque assay[11] and recombination confirmed by PCR. The supernatant from the positive plaques was used to inoculate insect cells seeded at 5×10^5 cells/ml in serum-free medium (200 μl/15 ml). The viral stock obtained after 5 days was used as innoculum for cells and tested after a further 5 days for recombinant C9. *Trichoplusia ni* (5BI-4) cells can produce 20-fold more recombinant protein than other insect cells.[12] We compared recombinant C9 production in these and *Spodoptera frugiperda* (IPLB-SF9 or Sf21) cells (R&D Systems). All cells were maintained in protein-free medium, excell 401, pH 6·2 at 27° (Sera Lab, Crawley Down, UK). Sf9 cells were grown in TNM-FH medium (Sigma, Poole, UK) supplemented with 10% fetal calf serum for co-transfection.

Generation of cDNA coding for C9 mutants

Three C9 mutants were engineered.

Mutant 1. C9 contains 24 cysteines (Fig. 1), present as disulphide bonds and thought to be important for C9 polymerization.[1] Two cysteine residues (33 and 36) were mutated to alanine, to alter the structure and polymerization characteristics of the protein. Two fragments were made by PCR using oligonucleotides C9(7) and C9(5) (Fig. 2, lane 4), and C9(8) and C9(11) (Fig. 2, lane 5). These fragments were joined by PCR with C9(12) and C9(13) (Fig. 2, lane 6).

Mutant 2. We had previously engineered an unglycosylated C9 mutant without the 23 N-terminal amino acids[7] that polymerized more readily than wilde-type C9. This mutant was engineered in insect cells using oligonucleotides C9(18) and C9(5) followed by oligonucleotides C9(12) and C9(13) (Fig. 2, lane 3).

Mutant 3. Three consensus sequences for calcium-binding proteins with EF-hands were used to test for homologous regions in C9 (Table 1) using Genepro computer software. One region (residues 101–112) in human C9 showed good homology. Four adjacent acidic residues (D-109, E-110, D-111 and D-112) were mutated to alanine to destroy any putative calcium- or cation-binding site. Two cDNA fragments were made using oligonucleotides C9(11) and C9(16), and C9(17) and C9(5). These fragments were joined by PCR with oligonucleotides C9(12) and C9(13) (Fig. 2, lane 2).

Purification of recombinant C9

The insect cell supernatant inhibited C9 lysis of pigeon erythrocytes (results not shown), which was removed using a

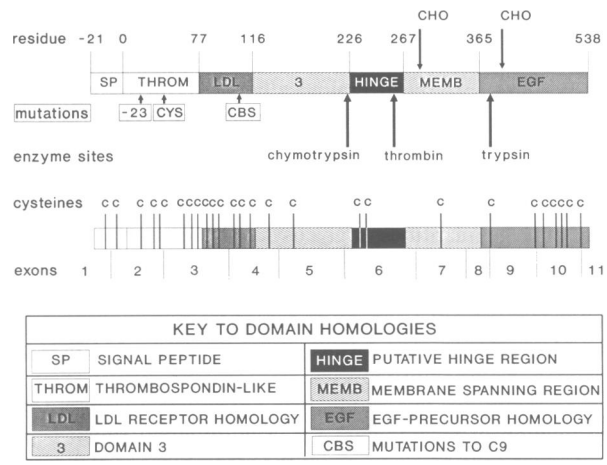

Figure 1. Structural characteristics of human complement component C9 and mutations. This figure illustrates the various domains in C9, the enzyme sites, the two N-linked carbohydrate side chains (CHO), the positions of the cysteine residues (C), the exons, and also the positions of the three mutations made to C9.

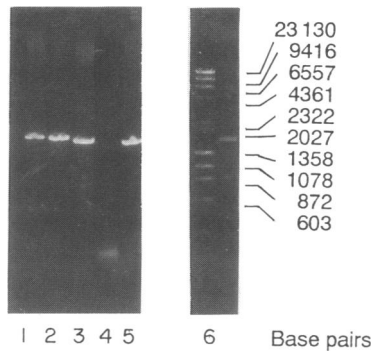

Figure 2. Agarose gel of PCR products. This figure shows cDNA encoding wild-type C9 (lane 1) after a two-stage PCR. It also shows the cDNA encoding three mutated C9s, the full-length cDNA coding for C9 with a mutated putative calcium-binding site (lane 2), cDNA for C9 lacking 23 N-terminal amino acids (lane 3), and a C9 mutant with two mutated cysteines (33 and 36) showing two fragments from one-stage PCR (lanes 4 and 5), and the full-length mutant cDNA after the second stage PCR (lane 6).

MonoQ HR 5/5 anion exchange column fitted to an FPLC system (Pharmacia LKB, Biotechnology, Uppsala, Sweden). The column was equilibrated with 10 mM sodium phosphate buffer, pH 7·4, and recombinant C9 eluted using a linear gradient to 0·5 M NaCl. The purity of C9 was increased by extending the gradient over the range 0·17–0·4 M and increasing the salt concentration by 0·01 M in 7 min. Recombinant C9 eluted at approximately 0·2 M NaCl and was 60% pure. The C9 was concentrated by centrifugation using Millipore ultrafree filtration units and stored at −70°.

Enzymatic cleavage, deglycosylation and Zn^{2+} polymerization of native and recombinant wild-type C9

C9 was cleaved by trypsin[17] and α-thrombin[18] as described previously and enzymatically deglycosylated by incubation of 2 μg with 0·4 U PNGaseF, 2·5 mU O-glycosidase and 2 mU neuraminidase in 100 μl of 0·25 M sodium phosphate buffer, pH 7·4, with 10 mM EDTA and 10 mM β-mercaptoethanol for 18 hr at 37°. Native and recombinant C9 were polymerized in the presence of Zn^{2+}, as previously described.[19]

SDS–PAGE and immunoblotting

Samples (20 μl) were analysed by 10% SDS–PAGE in reducing conditions (2·5% β-mercaptoethanol). Pellets were prepared by washing twice in phosphate-buffered saline (PBS) before incubation at room temperature with 300 μl PBS + 1% CHAPS to solubilize them. Protein bands were visualized by staining with Coomassie blue or immunoblotted onto nitrocellulose and compared to native C9 by densitometric scanning, as described previously.[7]

RESULTS

Expression and characterization of recombinant wild-type C9 from insect cells

The level of expression of recombinant C9 by three insect cell lines was assessed by immunoblotting aliquots of the insect cell supernatant daily over 7 days (Fig. 3). With the native signal peptide, *T. ni* cells produced most recombinant C9 [2·5 ± 0·9 (n = 7) μg/ml] but the yield was only twice that of the other cell lines. Further optimization of secretion was attempted by replacing the native signal peptide with the honey-bee melittin secretory signal peptide, previously shown to increase secretion of foreign proteins from insect cells fivefold.[20] This signal peptide doubled production of recombinant protein in both Sf9 [2·9 ± 2·2 (n = 12) μg/ml] and *T. ni* [5·0 ± 1·2 (n = 7) μg/ml] cells. Together, these modifications increased production of recombinant C9 from 1·3 ± 0·8 (n = 7) μg/ml (Sf9 cells with native signal peptide) to 5·0 ± 1·2 (n = 7) μg/ml (*T. ni* cells with honey-bee melittin signal peptide).

Table 1. Homology of human, mouse and trout C9 to EF-hand calcium-binding sites

Site 1	[DENTE]X[DNES]X[DSNGE]X[FTYKE]X[DSETG]XX[EDVNQ]		
Human	91	CIKMRLRCNG DNDCGDFSDE DDCESEPRPP CRDRVVEESE LARTAGYGIN ILGMDPLSTP	150
Mouse	91	IKRRLLCNGD NDCGDYSDEN DCDDDPRTPC RDRVAEESEL GLRAGYGINI LGMEPLRTPF	150
Trout	91	CIKLRLSCNG DYDCEDGSDE DCEPVRKPCG TKLYDTNEQG RTAGYGINIL GMEPRINPFN	150
Site 2	DX[DNS]X[DENSTG]XXX[DENQTSCGA]XX[DE]		
Human	91	CIKMRLRCNG DNDCGDFSDE DDCESEPRPP CRDRVVEESE LARTAGYGIN ILGMDPLSTP	150
Mouse	91	IKRRLLCNGD NDCGDYSDEN DCDDDPRTPC RDRVAEESEL GLRAGYGINI LGMEPLRTPF	150
Trout	91	CIKLRLSCNG DYDCEDGSDE DCEPVRKPCG TKLYDTNEQG RTAGYGINIL GMEPRINPFN	150
Site 3	[DENQST]X[DENQST]X[DENQST]X[DENQST]X[DENQST]XX[DENQST]XXR		
Human	91	CIKMRLRCNG DNDCGDFSDE DDCESEPRPP CRDRVVEESE LARTAGYGIN ILGMDPLSTP	150
Mouse	91	IKRRLLCNGD NDCGDYSDEN DCDDDPRTPC RDRVAEESEL GLRAGYGINI LGMEPLRTPF	150
Trout	91	CIKLRLSCNG DYDCEDGSDE DCEPVRKPCG TKLYDTNEQG RTAGYGINIL GMEPRINPFN	150

This table illustrates the homology of region 101–112 in human C9, 101–112 in mouse C9 and region 98–118 in trout C9 to three consensus sequences for EF-hand calcium-binding sites. Site 1 was obtained from the frequency of amino acids occurring at each position in the calcium-binding loop of 165 EF-hands,[13] where X can be any amino acid, and any of the amino acids in the square brackets are allowed in positions 1, 3, 5, 7, 9 and 12, which are the key co-ordinating residues in the calcium-binding loop. Site 2 was obtained from a consensus sequence of a divalent cation binding EF-hand motif found in calmodulin and parvalbumin.[14] In this sequence, [ILVFYW] at position 4 and [GP] at position 7 were unacceptable amino acids. Site 3 was obtained from a consensus sequence for 160 different EF-hand containing proteins.[15] This formula is modified for human and mouse C9 by the addition of an arginine residue three residues downstream, which has been shown to have a stabilizing effect on calcium-binding sites.[16] However, trout C9 did not posess this residue. There is also a high incidence of negatively charged amino acids (D and E) in this homologous region.

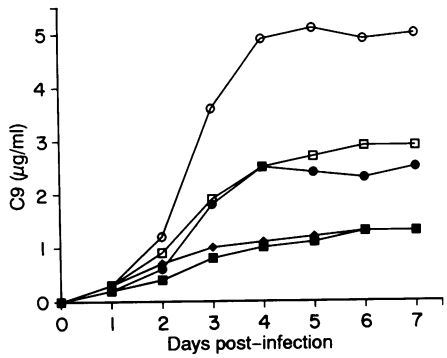

Figure 3. Time–course of secretion of recombinant wild-type C9 from insect cells. The secretion of recombinant wild-type C9 from three different insect cell lines is compared over a period of 7 days post-infection. C9 was measured in the insect cell supernatant by immunoblotting after 10% SDS–PAGE in reducing conditions and comparison to native C9 by densitometry. The amount of protein secreted when the native signal peptide was used was greater with *T. ni* cells (●) than both Sf9 (■) and Sf21 (◆) cells. The production of recombinant protein in both Sf9 cells (□) and *T. ni* cells (○) was doubled by using the honey-bee melittin signal peptide.

Recombinant wild-type C9 was characterized and compared to C9 from plasma using the following criteria: mobility on SDS–PAGE, reaction with various C9 antibodies, cleavage with trypsin and α-thrombin, enzymatic deglycosylation, polymerization in the presence of Zn^{2+}, and haemolytic activity.

The molecular weight of recombinant protein obtained with either signal peptide (65 000 MW; Fig. 4, lanes 2 and 3) was 6000 MW less than that of C9 extracted from plasma (71 000 MW; Fig. 4, lane 1). Recombinant protein reacted with all C9 antibodies (results not shown). Trypsin cleaves native C9 between residues 392–393,[1] producing two major fragments and many minor fragments.[18] Recombinant (lane 11) and native (lane 12) C9 both produced similar fragments when cleaved with trypsin (Fig. 5). Native C9 is cleaved by α-thrombin between residues 244–245[21] producing two fragments, one with no N-linked glycosyl chain and one larger fragment containing two N-linked glycosyl chains. When native (lane 9) and recombinant (lane 10) C9 were cut with α-thrombin (Fig. 5), the smaller fragments had the same mobility on SDS–PAGE whereas the larger fragments had a 6000 MW difference, the same as the full-length proteins. This provided further evidence that the difference in size observed between native and recombinant C9 was due to different glycosylation. This was confirmed by enzymatic deglycosylation of native and recombinant C9 (Fig. 5, lanes 1–6) to the same core size (Fig. 5, lanes 4 and 6). Native C9 from plasma and wild-type recombinant C9 both polymerized to the same extent in the presence of Zn^{2+} (Fig. 5, lanes 7 and 8) and had the same haemolytic activity (Fig. 6). The wild-type protein generated with either signal peptide had the same haemolytic activity (results not shown).

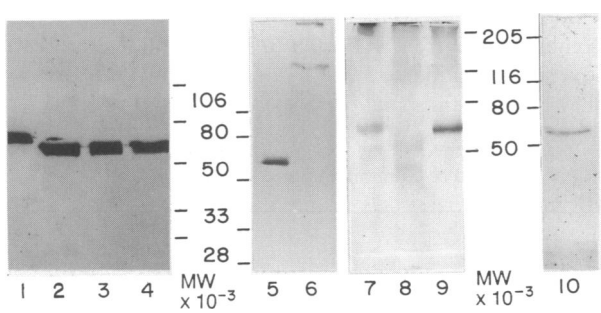

Figure 4. Immunoblot of recombinant and native C9 under reducing conditions. This figure shows results of immunoblotting the supernatants of insect cells that had been infected with the relevant C9 vector 5 days previously. Samples of supernatant (20 μl) were run on 10% SDS–PAGE in reducing conditions and compared to 100 ng of native C9 (lane 1) prepared from plasma (71 000 MW). Lane 2 shows recombinant C9 that had the native signal peptide (65 000 MW), lanes 3 and 5 show recombinant C9 that had the honey-bee melittin signal peptide (65 000 MW) and lane 4 shows the putative calcium-binding site mutant (65 000 MW). The mutant lacking the first 23 N-terminal amino acids appears as polymer in the supernatant (lane 6), although there is evidence of monomer present in the cell pellets (lane 9) when compared to the cell pellets of recombinant native C9 (lane 10) and the putative calcium-binding site mutant (lane 8). All pellet samples had some polymer, although none was evident in the supernatant for the wild-type or calcium-binding site mutant. The sample of C9 with mutated cysteines (33 and 36) was concentrated 50 times before it was detectable by immunoblotting (65 000 MW, lane 7). The cell pellets were solubilized as detailed in the Materials and Methods.

Characterization of three C9 mutants

C9 with a mutated putative calcium-binding site (residues 109–112) was secreted to the same extent by *T. ni* cells [4·53 ± 1·9 (n = 3) μg/ml], had the same mobility of 65 000 MW on SDS–PAGE (Fig. 4, lane 4), deglycosylated to the same core size (Fig. 5, lane 5) and had the same haemolytic activity (Fig. 6) as wild-type recombinant C9. It also reacted with all C9 antibodies and did not polymerize in the insect cell supernatant. C9 without 23 N-terminal amino acids was secreted to a lesser degree than wild-type recombinant C9 (approximately 0·6 μg/ml), appearing as a polymer in the insect cell supernatant (Fig. 4, lane 6), rendering it haemolytically inactive (results not shown). However, there was evidence of the presence of a monomer of the correct size for recombinant C9 without 23 N-terminal amino acids (62 000 MW) in the cell pellets (Fig. 4, lane 9), indicating that the mutation may have increased its tendency to polymerize. The third mutant, with cysteines 33 and 36 mutated to alanines, was secreted poorly and after concentrating the supernatant 50 times a band of 65 000 MW was observed (Fig. 4, lane 7). It was not possible to demonstrate haemolytic activity of this mutant, presumably due to inhibition by the concentrated insect cell supernatant.

DISCUSSION

The results reported here show that, using a baculovirus insect cell system, it is possible to make large quantities of recombinant wild-type and mutant C9 with the same biological characteristics as native C9 and produce mutants that have altered polymerization. Increased secretion of recombinant C9 was possible using the honey-bee melittin signal peptide in conjunction with *T. ni* cells, producing 5 μg/ml insect cell supernatant, sevenfold greater than the previously reported yield of C9 from insect cells (0·7 μg/ml) and 50-fold greater than the yield of C9 (0·11 μg/ml) from COS-7 cells.[8] The wild-type recombinant C9 was equivalent to C9 from plasma, as judged by haemolytic activity, reaction with C9 antibodies, trypsin and

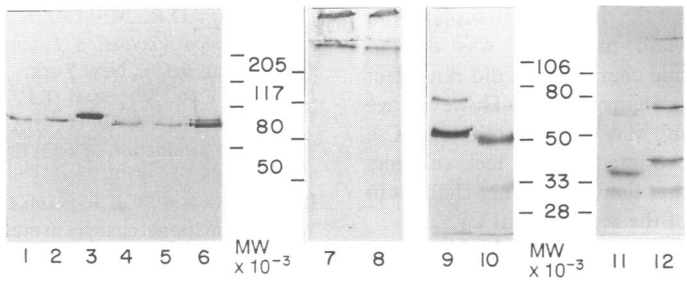

Figure 5. Enzymatic deglycosylation, Zn^{2+} polymerization and enzymatic cleavage of recombinant and native C9 under reducing conditions. This figure shows the results of immunoblotting samples of native and recombinant C9 that had either been enzymatically deglycosylated, cleaved with trypsin or α-thrombin, or polymerized in the presence of Zn^{2+}. All samples were run on 10% SDS–PAGE. Lanes 1–6 compare the bands obtained before and after enzymatic deglycosylation: native C9 before (71 000 MW; lane 3) and after (61 000 MW; lane 6); wild-type recombinant C9 before (65 000 MW; lane 1) and after (61 000 MW; lane 4); and recombinant calcium binding-site mutant C9 before (65 000 MW; lane 2) and after (61 000 MW; lane 5). All recombinant proteins had the same mobility after deglycosylation (lanes 4–6). Native C9 (lane 7) and recombinant wild-type C9 (lane 8) both polymerized to the same extent after incubation in the presence of Zn^{2+} (Coomassie stain). The effect of α-thrombin on native C9 (lane 9) and recombinant wild-type C9 (lane 10) was comparable. The smaller N-terminal fragment with no N-linked glycosyl chains had the same MW, whereas the larger C-terminal fragment with both N-linked chains had a difference of 6000 MW, the same as the full-length proteins which were also present in these samples. Trypsin cut both native C9 (lane 12) and recombinant wild-type C9 (lane 11) to produce a similar pattern of bands in both lanes.

α-thrombin cleavage, ability to polymerize in the presence of Zn^{2+} and enzymatic deglycosylation to the same core size.

There are three groups of calcium-binding proteins; those with EF-hands, calcium lipid-binding proteins and proteins that have post-translationally modified glutamic acid residues.[22] The EF-hand group contains a 12-residue calcium-binding loop flanked on either side by an α-helix in a helix–loop–helix conformation. Calcium binds to these proteins inducing structural changes which expose hydrophilic surfaces that can attach to target proteins.[22] Three types of consensus sequence have emerged from analysis of numerous calcium-binding proteins with EF-hand loops (Table 1) that were used to test for homologous regions in C9. A region in human C9 (residues 101–112), in mouse C9 (101–112) and trout C9 (98–118) containing a large proportion of negatively charged residues show good homology to these sequences (Table 1). Human and mouse C9 also exhibit a strong α-helix potential following this region and a weak one preceding it, using the formula of Chou & Fasman.[23] This putative calcium-binding site was mutated by changing residues 109–112 to alanines and, after expression in insect cells, was found to be equivalent to wild-type recombinant C9. This mutation did not affect the haemolytic activity of the protein, suggesting that this site was not involved in haemolysis. The earliest detectable intracellular response of nucleated cells to sublytic attack by the MAC, formed by the polymerization of C9, is a rapid increase in intracellular calcium.[24] C9 can polymerize in the presence of divalent cations,[19] especially Zn^{2+}, and has also been shown to bind one mole of calcium per mole of C9.[25] However, since the thresholds for haemolysis and polymerization of C9 occur at different times,[3] it is now necessary to test the effect of this mutation on the recovery mechanism of nucleated cells.

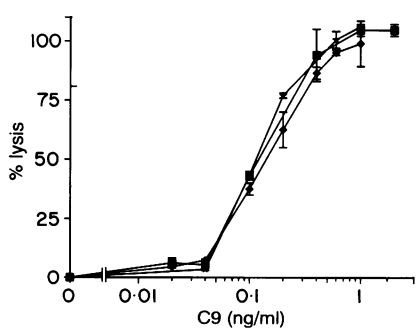

Figure 6. Haemolytic activity of native and recombinant C9. This figure compares the haemolytic activity of recombinant and native C9, as judged by lysis of pigeon erythrocytes bearing preformed C5b-8 sites. The specific activity ($C9H_{50}$) of wild-type recombinant C9 (0·14 ng/ml; ◆) and the putative calcium-binding site (CBS) mutant (0·11 ng/ml; ★) was comparable to that of native C9 extracted from human plasma (0·12 ng/ml; ■).

Another mutant, lacking 23 N-terminal amino acids, had increased polymerization, which may prove useful for investigating the effect of C9 polymerization on the recovery mechanism. An antibody to a peptide (residues 11–28 of human C9) inhibits C9 polymerization, but does not react with native monomeric protein or C9 in the MAC, suggesting that this section of C9 unfolds prior to polymerization.[26] Previously we have constructed an unglycosylated mutant of C9 without the 23 N-terminal amino acids[7] which produced fourfold more polymer than wild-type C9, implying that residues 1–23 are involved in stabilizing the protein and preventing spontaneous polymerization. A third mutant, cysteines 33 and 36 mutated to alanines, was produced 100-fold less than wild-type C9, making it difficult to characterize. The question therefore arises whether this mutation altered the secretion of the protein by degrading it in the cell, as has been reported with some C9 mutants in COS-7 cells.[27]

Insect cells cannot produce complex carbohydrate chains[28] such as those present in C9, nor add terminal sialic acid residues to proteins.[28,29] C9 activity is not affected by removal of terminal sialic acid residues, which account for 4000 MW.[30] It has been shown recently that insect cells can process complex carbohydrates[31] after infection with baculovirus which activates the relevant enzymes.[32] However, we found no change in the MW of recombinant C9 with time after infection (results

not shown). Our results show that the difference in mobility on SDS–PAGE of recombinant and native C9 was due to differences in the glycosylated side chains which did not affect the haemolytic activity of the proteins (Fig. 6). However, we observed a 6000 reduction in MW of recombinant C9, 2000 MW greater than that accounted for by lack of sialic acid residues alone, suggesting that there were other changes in the carbohydrate composition of the recombinant C9.

The results presented here show that the baculovirus insect cell system can generate large quantities of recombinant and mutant C9 and that C9 mutants with altered polymerization capacity can be engineered, both from the baculovirus expression system reported here, and from previous work using an *in vitro* method.[7] This will enable our hypothesis concerning the thresholds involved in the molecular sequence of C9 insertion in the membrane,[3] in particular the effect of C9 polymerization on the removal of the MAC by vesiculation, to be tested.

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