Molecular basis for complement component 6 (C6) deficiency in rats and mice

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Summary

Complement component C6 is a part of the lytic membrane attack complex formed during complement activation. Animal modeling to define the role of C5a vs. C5b-9 in human disease has used rodents deficient in C6, yet the molecular basis for the deficiencies has not been ascertained. Oligonucleotides derived from a 493 bp EST sequence of the rat C6 gene were used to isolate full-length transcripts of rat C6 mRNA. Sequence analysis confirmed that the derived amino acid sequence for rat C6 is highly homologous to human and mouse. We identified a 31 bp deletion in exon 10 of the C6 gene that leads to C6 deficiency in a strain of PVG rats (PVG/c-) and developed a PCR-based genotyping test. In addition, we identified four point mutations in the mouse C6 gene that may result in C6 deficiency observed in the Peru-Coppock mouse strain. A serendipitous finding from this study was a coagulation defect in the C6 deficient mice and rats. C6 deficient mice or rats demonstrated prolonged tail bleeding times that was reversed by treatment with purified rat C6 protein. Further, adenosine diphosphate induced platelet aggregation were markedly reduced in C6 deficient rats. The molecular basis for these coagulations defects is unknown at present.

Keywords: Cloning; C5b-9; Membrane attack complex

Introduction

The complement system forms an activation cascade of proteins with similar organization and complexity as the blood clotting system. Complement may be activated via three distinct pathways; classical, lectin and alternative pathway. Following activation and subsequent formation of enzymatic complexes called C3 and C5 convertases, the pathways enter a common terminal cytolytic pathway which results in the interaction of five complement proteins (i.e., C5b, C6, C7, C8 and C9) to form the membrane attack complex (MAC), C5b-9.

Various animal models and inhibitory studies have demonstrated the importance of the terminal complement components in models of human disease. Chance observations led to the discovery of a strain of PVG rats (PVG/c-) that were deficient in the complement component C6 and unable to form the C5b-9 complex (Leenaerts et al., 1994; van Dixhoorn et al., 1997). A mutation-prone Peru-Coppock mouse strain deficient in complement component C6 also is unable to form the C5b-9 complex and apparently does not produce C6 protein (Orren et al., 1989). These C6 deficient rodents have been used extensively to study the role of complement in various disease models (Brandt et al.,...
Characterization of the rat C6 deficiency concluded that the loss of C6 was probably due to a hereditary point mutation in the C6 gene or the formation of an unstable transcript (van Dixhoorn et al., 1997). However, the exact nature of the mutation was not determined. Identification of the mutation was further hampered by the fact that the rat C6 gene was not cloned. Similarly, the molecular characterization of C6 deficiency in the mouse has not been characterized. In the present study, we have cloned rat C6 gene and identified the molecular basis for C6 deficiencies in these rat and mouse strains.

Materials and methods

cDNA cloning and sequencing

Rat C6 study
Liver total RNA from PVG/c- or PVG rats was isolated by TRIzol reagent (Invitrogen Corp, Carlsbad, CA). First strand cDNA was generated using SMART RACE cDNA kit (BD Bioscience, Palo Alto, CA). Primers utilized for PCR reactions to obtain the wild type rat C6 cDNA sequence were, UPM 3' RACE (BD Bioscience) and the primers listed in Table 1 (WT Rat primers). Primers utilized for PCR reactions to obtain the C6 cDNA sequence from PVG/c- rats were the same as for WT plus additional primers as outlined in Table 1 (PVG/c- Rat primers). All PCR reactions (35 cycles) were performed using the Advantage 2 PCR system (BD Bioscience) with annealing at 62°C. All PCR products were subcloned into PCR-4 TOPO vector (Invitrogen) and sequenced at the Core facility (MBCF, Dana Farber Cancer Institute, Boston, MA).

Genomic DNA from rat tails was prepared by proteinase K degradation of tail biopsies. Genotyping was performed by PCR (35 cycles) using primers listed in Table 1 (Rat C6 genotyping primers) with annealing at 55°C.

Mouse C6 study
Mouse liver total RNA was prepared as described above. First strand cDNA was prepared using Reverse Transcription System (Promega, Madison, WI). PCR

Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Type</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>WT Rat primers</td>
<td>5’ GSP (5’-CTCGATGGGCCAGCAACAACAAAGC-3’)</td>
</tr>
<tr>
<td></td>
<td>3’ GSP (5’-CCGAGTGTCCCTAAACCAGTGTTCA-3’)</td>
</tr>
<tr>
<td></td>
<td>C6 5’ (5’-AAGGCATGACCGACATCCTAC-3’)</td>
</tr>
<tr>
<td></td>
<td>C6 3’ (5’-CACCCTTCCACACTTCTCGTGCG-3’)</td>
</tr>
<tr>
<td>PVG/c- Rat primers</td>
<td>Internal 1 (5’-CCTGGAGGCGCATCAATGCCTC-3’)</td>
</tr>
<tr>
<td></td>
<td>Internal 2 (5’-AGCCGCCAGAGCTACAGAACT-3’)</td>
</tr>
<tr>
<td></td>
<td>Internal 3 (5’-GAGTGGTTAGAGTCGGTGAAGG-3’)</td>
</tr>
<tr>
<td></td>
<td>Internal 4 (5’-TTTCTGCGCAGGAGGCCCAAGAG-3’)</td>
</tr>
<tr>
<td></td>
<td>Internal 11 (5’-CCTGGGGCTTGGCTCAGGGTTA-3’)</td>
</tr>
<tr>
<td></td>
<td>Internal 12 (5’-CTGAGGGTAAATATGTTGGTTCA-3’)</td>
</tr>
<tr>
<td>Rat C6 mut-1</td>
<td>(5’-GCAATGTGCTCCATGTCCT-3’)</td>
</tr>
<tr>
<td>C6 mut-3</td>
<td>(5’-GGTCCCCAGATTATAAATCG-3’)</td>
</tr>
<tr>
<td>Mouse C6 primers</td>
<td>5’ (5’-GGACGGCTTTGGATGCTCACACA-3’)</td>
</tr>
<tr>
<td></td>
<td>3’ (5’-CTCAGGCACAGCTGCTCTTT-3’)</td>
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Fig. 1. Rat C6 cloning strategy. Rat C6 cDNA was cloned in two fragments. Primers 2 and 4 were used in a RACE reaction using the Clontech RACE kit to obtain a ~1 kb fragment. The cDNA template created with the RACE kit was then used as a template in a PCR reaction using primers 1 and 3 to obtain a ~2.4 kb fragment. The fragments were sequenced to obtain the complete cDNA sequence. See Table 1 for primer sequences.

Fig. 2. Nucleotide and amino acid sequence of rat C6. (A) The cDNA sequence of C6 includes the complete coding region, complete 3' UTR and partial 5' UTR. Deduced amino acid sequence is shown by standard single letter symbols beneath the cDNA sequence. The numbers on the left indicate the nucleotide bases and amino acid residues (italicized numbers). Peptide fragments in boxes were confirmed by LC-MS/MS. (B) Comparison of the rat C6 cDNA sequence with the rat genome database (Ratmap) reveals that the rat C6 gene is comprised of 17 exons and 16 introns spanning 71.7 kb of genomic DNA. The size of introns and exons are shown in base pairs (bp).
was carried out using primers listed in Table 1 (Mouse C6 primers).

**Purification of rat C6 protein**

Rat C6 was purified using a polyclonal immunoaffinity column. Rat serum was diluted 1:3 with PBS-EDTA-NaCl buffer (750 mM NaCl, 10 mM EDTA, 1 × PBS, 0.01% sodium azide, 50 mM benzamidine) and loaded on the column. The column was washed with PBS-EDTA-NaCl buffer. C6 protein was eluted with 100 mM glycine (pH 3.0) and 1 ml fractions were collected. Pooled protein fractions were dialyzed overnight in PBS at 4°C and subsequently concentrated. Coomassie blue staining of this preparation run on a SDS-PAGE gel showed that the majority of eluted protein was a single band at the appropriate molecular size (100 kDa).

**Hemolysis assay**

Human serum, C6 depleted human serum (Advanced Research Technologies) and C6 depleted human serum supplemented with purified rat C6 were serially diluted 1:2 (20% serum to 0.156% serum) in GVB²⁺ buffer (gelatin veronal–buffered saline: 0.1% gelatin, 141 mM NaCl, 0.5 mM MgCl₂, 0.15 mM CaCl₂, 1.8 mM sodium barbital) and added in duplicate (100 µl/well) to a 96-well plate. The plate was then incubated at room temperature for 30 min. Chicken erythrocytes (RBC; 5 × 10⁷/ml in 4 ml of GVB²⁺) were sensitized with anti-chicken RBC polyclonal antibody (Intercell Technologies, 0.1% v:v) and incubated at 4°C for 15 min. The RBC were washed twice with GVB²⁺ and resuspended to a final volume of 2.4 ml in GVB²⁺. The RBC (30 µl/well, 2.5 × 10⁶ cells) were added to the plate containing serum and rat C6, mixed well and incubated at 37°C for 30 min. The plate was then centrifuged at 1000g for 2 min, and 85 µl of the supernatant was transferred to a new 96-well microtiter plate. The plate was read at 415 nm with a microplate reader, and the percent serum complement hemolytic activity was determined by the following formula (where OD is optical density): % hemolysis = 100 × [(OD sample–OD GVB²⁺ Control)/(OD 100% lysed control–OD GVB²⁺ Control)], where 100% lysed control is lysis obtained by addition of 100 µl GVB²⁺ containing 1% Triton to the 30 µl of chicken RBC as prepared above.

**Tail bleeding times**

Tail bleeding times were measured as previously described (Sambrano et al., 2001). Briefly, mice aged 6–8 weeks were anesthetized with a mixture of ketamine and xylazine, and tails were transected 2 mm from the tip with a scalpel blade. The bleeding end was immersed in PBS maintained at 37°C and the time required for stoppage of blood flow (stoppage for 60 s or more) was recorded. Assays were terminated at 15 min. Reconstitution of C6 in C6 deficient mice was done by injection of 60 mg of purified rat C6 via the penile vein.

**Platelet aggregation**

Platelet aggregation was performed as we have described previously (Lefer et al., 1988). Briefly, blood from rats was collected in a syringe containing sodium citrate (3.9% w/v, 9:1 v/v) and centrifuged at 200g for 15 min. The top layer, platelet rich plasma (PRP), was
removed and the remaining sample was centrifuged at 2000g for 20 min to obtain the platelet poor plasma (PPP). Platelet counts in PRP were adjusted to 2 x 10^6 cells/ml with PPP. Platelet aggregation was monitored using a turbidimetric aggregometer (Chronolog Corp, Haverton, PA) with samples stirred constantly at 1000 rpm and 37°C. Platelet aggregation was induced using ADP (Chronolog Corp, Haverton, PA).

**Statistical methods**

Data are represented as mean±SEM. Statistical comparisons of groups were made with one-way ANOVAs followed by posthoc analysis. Differences were considered statistically significant at a value of p≤0.05.

**Results**

**Cloning of rat C6 cDNA**

The rat C6 cDNA was cloned based on the homology between human and mouse C6 genes. BLAST searches of Genbank using human and mouse C6 cDNA sequences identified a 393 bp expressed sequence tag (EST), UI-R-C1-00-0-UI, from the University of Iowa rat EST project. Two gene specific oligonucleotides, 5’ GSP and 3’ GSP, were designed based on the EST sequence. RT-PCR reactions utilizing rat 3’ GSP primer, Clontech UPM 3’ RACE primer, rat 5’ GSP primer and rat C6 5’ primer (designed from conserved human and mouse C6 sequences) generated partially overlapping 2.4 and 1.0 kb fragments, respectively (Fig. 1). The rat C6 cDNA sequence was assembled from the partially overlapping sequences of the two fragments. Full length rat C6 cDNA was then obtained using the rat C6 5’ primer and the rat C6 3’ primer (designed based on the sequence of the 1.0 kb fragment).

**Nucleotide sequence of rat C6**

The complete coding region of the rat cDNA is contained within our full-length cDNA transcript of 2805 nucleotides (Accession #AY230250, Fig. 2A), which includes the previously described 492 bp cDNA fragment (Van Dixhoorn et al., 1997). The complete 3’ untranslated region, which was obtained by 3’ RACE,
extends for 201 bp after the stop codon. We were unable to obtain a distinct product using 5′ RACE and therefore were only able to amplify a part of the 5′ untranslated region utilizing a consensus primer based on the 5′ untranslated region from the human and mouse C6 sequence. BLAST search of the rat genome project (RATMAP) using a fragment of the cDNA sequence, identified a genomic contig from chromosome 2 which contained the entire genomic rat C6 sequence. Alignment of the cDNA sequence with the genomic sequence allowed us to identify the exon–intron boundaries. The rat C6 gene (Accession #AY343914) is composed of 17 exons and 16 introns spanning ~71.7 kb of genomic DNA (Fig. 2B). The exons range from 91 to 381 bp in length, whereas the introns range from 312 to 14047 bp in length. The cytogenetic location of the gene is 2q16.

Derived amino acid sequence of rat C6

The predicted amino acid sequence of rat C6 is highly homologous to the amino acid sequences of human and mouse C6 (Fig. 3). As expected for a secreted protein, the first 21 residues in the amino acid sequence have the typical features of a signal peptide, a 13-residue stretch of mainly hydrophobic residues flanked by positively charged residues on either side, which is most likely cleaved after the alanine residue. The mature peptide after cleavage of the signal sequence is composed of 913 residues with a calculated molecular mass of 102 kDa before any modifications. We purified rat C6 with an apparent molecular mass of approximately 80 kDa under non-reduced conditions (118 kDa under reduced conditions; data not shown) by affinity chromatography using polyclonal antibody against human C6. This band was excised and sent for micro-sequencing by liquid chromatography tandem mass spectrometry (LC-MS/MS), which confirmed four regions of the derived amino acid sequence (see Fig. 2A), and has a high degree of homology with human (84%) and mouse (90%) complement C6. After removal of contaminating rat IgGs (upper band in the “before” lane in Fig. 4A), we used the purified rat C6 (only band present in the “after” lane in Fig. 4A) in a hemolytic assay using C6 deficient human sera and sensitized chicken RBCs. Supplementation of C6 deficient human sera with the appropriate amount of rat C6 (i.e., 10% normal human sera contains 6 μg C6/ml) restored hemolytic activity (Fig. 4B).

Identification of mutation leading to C6 deficiency in PVG/c- rats

A chance observation that a strain of PVG rats lacked CH50 activity led to the discovery of C6 deficient PVG/c- rats (Leenaerts et al., 1994). It was postulated that these rats may carry a hereditary mutation which leads to the loss of C6 protein and an approximately 100-fold decrease in C6 transcript levels (van Dixhoorn et al., 1997), but the exact nature of the mutation was unknown. Quantitative real time RT-PCR demonstrated that the level of liver C6 transcript in C6 deficient rats was reduced by 150-fold compared to wild
type PVG rats (data not shown). Due to the extremely low levels of C6 transcript we were unable to amplify the full-length mutant C6 transcript from the PVG/c- rats by RT-PCR. Therefore, five sets of internal primers (see Table 1) were designed to obtain five partially overlapping RT-PCR products, which were sequenced and assembled to obtain the complete C6 cDNA sequence from PVG/c- rats (Fig. 1). Alignment of the PVG/c- and PVG C6 cDNA sequences identified a 31 bp deletion in exon 10 in C6 PVG/c- cDNA (Fig. 5A). This deletion leads to a frame shift in the reading frame and causes premature termination of translation 194 bp downstream of the deletion. Primers (Table 1) flanking the 31 bp deletion site were designed to verify the deletion and provide a rapid PCR test for genotyping PVG/c- rats using genomic DNA isolated from tails. As expected, the PCR product obtained using these primers is smaller from PVG/c- rats compared to PVG rats (Fig. 5B).

Identification of mutations leading to C6 deficiency in a strain of C3H/He mice

C6 deficient mice were derived from a Peruvian strain backcrossed with C3H/He mice for 10 generations (Orren et al., 1989). These mice lack functional C6 and antigenic activity but the molecular basis of the deficiency is unknown. A set of primers was designed based on the mouse C6 cDNA sequence from Balb/c mice (Genbank NM_016704) to amplify full length cDNA from wild type C3H/He and C6 deficient mice by RT-PCR. The RT-PCR products were ~2.2 kb in length and of equal intensity indicating that there is no reduction in the level of C6 transcript in the deficient mice unlike that observed in the PVG/c- rats (data not shown and confirmed by quantitative real time RT-PCR). Alignment of the cDNA sequences from wild type C3H/He and C6 deficient mice identified seven individual bp point mutations in C6 deficient mice (Fig. 6A). Four of these point mutations yield amino acid substitutions, which may result in C6 deficiency (Fig. 6B).

C6 deficiency affects coagulation in rats and mice

A serendipitous finding from these studies was a coagulation defect in C6 deficient rats and mice. While obtaining tail samples for our genomic studies we observed that the deficient animals tended to have prolonged bleeding compared to their wild type counterparts. Our PRP study from C6 deficient rats revealed that ADP induced platelet aggregation is markedly reduced in PVG/c- rats compared to wild type PVG rats (Fig. 7A). There was no detectable difference in maximum aggregation of platelets from PVG and PVG/c- rats upon stimulation with 10 μM ADP; however, stimulation with 5 or 2 μM ADP exhibited a significant reduction in maximum aggregation. Platelets from wild type PVG rats treated with an inhibitory anti-C5 monoclonal antibody (18A; 100 μg/ml) (Vakeva et al., 1998) reveal a similar significant reduction in maximal aggregation compared to untreated platelets (Fig. 7B). However, treatment of human PRP with
anti-C5 monoclonal antibody (5G1.1; 20 μg/ml) had no effect on ADP induced aggregation (Fig. 7C).

We also assessed the effect of C6 deficiency on tail bleeding time in C6 deficient mice. C6 deficient mice had prolonged bleeding times compared to wild type C3H/He mice (p < 0.05; Fig. 7D). The prolonged bleeding time observed in C6 deficient mice was reversed by pretreatment with purified rat C6 (7 mg/g body weight) (p < 0.05 compared to C6 deficient).

**Discussion**

The C6 gene in the present study encodes the second component of the rat terminal complement activation route that has been cloned and completely sequenced. The first terminal activation pathway component of the rat complement system characterized was C9 (Accession #U52948). Rat C5, C7 and C8 genes remain to be cloned; however, partial sequences of C5 (C5a) and C7...
are already present within Genbank. In addition, the recent completion of the rat genome project (Rat Genome Sequencing Project Consortium, 2004) simplifies the identification of the genomic structure of these genes. Availability of the complete sequences of all components of rat MAC will facilitate expression of recombinant proteins allowing the development of novel inhibitory monoclonal antibodies against these proteins and the study of structure and function interactions between the C5b-9 components.

The discovery of PVG/c- rats, which are deficient in C6, has proven to be extremely important as this is the only inbred complement deficient rat currently available for investigators to study the role of complement in various disease models. A previous study undertaken to characterize this deficiency concluded that the PVG/c rats showed an approximate 100-fold reduction in the level of C6 transcript and that the size of the C6 mRNA (e.g., northern blot) from PVG/c- rats was identical to the C6 mRNA from control PVG rats (Van Dixhoorn et al., 1997). In this study, we have identified a small 31 bp deletion in exon 10 of the C6 gene from PVG/c- rats, which leads to disruption of the open reading frame and confirmed a 150-fold reduction in C6 mRNA levels using quantitative real time RT-PCR. It is possible that the deletion leads to formation of an unstable mRNA, which in combination with production of a truncated peptide that is likely rapidly degraded, induces C6 deficiency. We have also developed a simple PCR test for genotyping PVG/c- rats using tail DNA that will allow the backcrossing of this deficiency onto other genetic backgrounds. Further, use of C6 deficient animals in conjunction with the inhibition of C5 with a monoclonal anti-rat C5 monoclonal antibody (Vakeva et al., 1998) will allow careful investigation of the role of C5a vs. C5b-9 complex in a variety of disease models. The added ease of production of native or recombinant rat C6 will also allow restoration of the C5b-9 pathway to confirm the roles of C5a and C5b-9 in disease models.

A C3H/He C6 deficient mouse strain was established more than a decade ago (Orren et al., 1989). We have identified seven point mutations in the C6 gene from these animals, four of which lead to amino acid substitutions possibly disrupting the tertiary structure of the protein causing the deficiency. It is also probable that, the amino acid substitutions interfere with the binding of C6 with either C5b or C7 thus inhibiting the formation of C5b-9. We are currently developing a SNP test for genotyping the C6 deficient mice in order to be able to cross the deficiency onto other genetic backgrounds. The use of anti-mouse C5 monoclonal antibody (Zhao et al., 2002; Zhou et al., 2000) can be used along with the C6 deficient mice to delineate the role of C5a vs. C5b-9 in disease models.

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We have also shown that the C6 deficiency in rats or mice leads to a defect in coagulation. PRP from C6 deficient rats revealed decreased platelet aggregation upon stimulation with ADP. Treatment of WT PRP with inhibitory anti-rat C5 antibody also induced a similar significant reduction in platelet aggregation. Furthermore, C6 deficient mice exhibited increased tail bleeding times that could be reversed by restoration with purified rat C6 protein. These data suggest a role for the MAC or a C5b-6 complex in coagulation. These studies are similar in scope to observations revealed in altered platelet aggregation studies in C6 deficient rabbits (Christian and Gordon, 1975) and humans (Wautier et al., 1979). This would not be surprising since multiple components of the coagulation and complement cascades interact with each other. However, our results indicate that inhibition of C5 does not alter aggregation of human PRP. Hence, it is possible that the dependence of platelet aggregation on terminal complement components might be limited to rodents.

In summary, we have cloned and sequenced the rat C6 gene. Identification of the molecular basis of the C6 deficiencies observed in rats and mice may aid in the development of novel tools for the study of the C5b-9 complex in models of disease. We observed altered bleeding profiles in these C6 deficient strains, however, the molecular basis for this phenotypic effect, remains to be elucidated.

References


