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Molecular characterization of GcC8 alpha, the functional homologue of human C8 alpha in the shark, Ginglymostoma cirratum

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MOLECULAR CHARACTERIZATION OF GcC8 ALPHA, THE FUNCTIONAL

HOMOLOGUE OF HUMAN C8 ALPHA IN THE SHARK, *GINGLYMOSTOMA*

CIRRATUM

A thesis submitted in partial fulfillment of the

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Lydia Tatiana Aybar

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To: Dean Kenneth Furton
   College of Arts and Sciences

This thesis, written by Lydia Tatiana Aybar, and entitled Molecular Characterization of GcC8 alpha, the Functional Homologue of Human C8 alpha in the Shark, *Ginglymostoma cirratum*, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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The thesis of Lydia Tatiana Aybar is approved.

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Dean Kenneth Furton
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Interim Dean Kevin O'Shea
University Graduate School

Florida International University, 2010

ABSTRACT OF THE THESIS

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Professor Charles H. Bigger, Major Professor

The focus of this study is to elucidate the components of the nurse shark

(*Ginglymostoma cirratum*) membrane attack complex (MAC), specifically complement

component C8α (GcC8α). Nurse shark C8α gene was cloned, sequenced, and analyzed

and Western blot analysis performed to identify components of shark MAC. GcC8α

consists of 2341 nucleotides that translate into a 589 amino acid sequence that shares

41.1% and 47.4 % identity with human and xenopus C8α, respectively. GcC8α conserves

the MAC modular architecture and cysteine-rich backbone characteristic of complement

proteins, including the cysteine residue that forms the C8α-γ bond as well as the indel

that is unique to C8α. Conservation of MAC protein structure is evident from cross-

reactivity of antihuman-MAC antibodies with shark serum proteins in Western blots

which confirmed the presence of C8 and C9-like proteins in shark serum, however, did

not resolve the question of whether C6 and/or C7 like proteins are present in shark.

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CHAPTER I.

INTRODUCTION

The focus of this study is to elucidate the components of the shark membrane attack complex (MAC), specifically its C8α ortholog. To further our understanding of the evolution of the complement system, it is essential that components of the cascade be defined, specifically the characterization of the genes that encode the key proteins like those involved in membrane attack. As a first step in this endeavor, the nurse shark C8α gene was cloned, sequenced, and analyzed and Western blot analysis was performed to assess individual constituents of shark MAC, the end product of the terminal pathway of complement.

The complement system is a group of heat labile serum proteins that are generally synthesized by the liver and that circulate as inactive precursors (with the exception of factor D). Complement activation occurs via three distinct activation pathways: the classical (CP), the alternative (AP), or the lectin (LP) (Muller-Eberhard, 1988; Volanakis, 1998; Lambris et al., 1999). All three activation pathways converge into a single terminal lytic pathway leading to the formation of MAC (Podack, 1988). MAC glycoproteins, C5b-C9, assemble sequentially, penetrate target cell membranes, and cause cell death (Podack, 1988). Complement genes, including those that encode the MAC proteins, are believed to have been generated by gene duplication events (Ohno, 1970; Nonaka et al, 1994). This view point is supported by the similar modular architecture of MAC proteins (C6 through C9) and that they are more similar to one another than they are to genes of proteins composing the activation pathways of the

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complement system (CP, AP, LP), although all complement proteins do share some common structural motifs. The mammalian MAC family of proteins is structurally composed of combinations of several modules or domains such as the thrombospondin type I (T1), low-density lipoprotein receptor class A (LA), epidermal growth factor precursor (EG) modules, and a large MAC-perforin (MACPF) domain (Figure 1) (Gonzalez et al., 1996). These modules are conserved in mammals (Gonzalez et al., 1996); amphibians (unpublished – genbank sequences); and teleosts (Papanastasiou and Zakardis, 2005; 2006a and b). This study provides evidence of a MAC gene in an elasmobranch, the nurse shark *Ginglymostoma cirratum*, the most basal organism to have a gene that is characteristic of the MAC family. This study further shows that the key MAC structural modules, T1, LA, EG, and MACPF, are present in the shark and suggests functional similarity of shark MAC to that of its mammalian homologue and a degree of similar antigenic structure since antibodies to human C8 and C9 cross react with shark C8 and C9 in Western blots.

The lytic activity of the nurse shark (*Ginglymostoma cirratum*) complement system involves functional analogues of mammalian C8 and C9 (Jensen et al., 1973; 1981). This report is the first to describe the human C8α subunit ortholog in the shark, GcC8α. The GcC8α gene consists of 2341 nucleotides that translate into a 589 amino acid sequence that shares 41.1% and 47.4 % identity with human and xenopus C8α, respectively. GcC8α conserves the MAC modular architecture and cysteine-rich peptide backbone characteristic of complement proteins, including the cysteine residue that forms the C8α-γ bond. Southern blot analysis shows that GcC8α exists as a single-copy gene

expressed in most tissues with the liver being the main site of synthesis. To further prove

that GcC8α is a C8α ortholog, phylogenetic analysis places it in a clade with other C8α

orthologs and as a sister taxa to *Xenopus*. The hydrophobicity profile of GcC8α is

consistent with the human C8α hydrophobicity pattern with the presence of hydrophobic

residues essential for membrane insertion. The conservation of structure and possibly

function of MAC proteins is supported by Western blot analysis of shark serum which

shows proteins cross-reactive with human anti-MAC antibodies.

This study begins to elucidate shark MAC, specifically its C8α ortholog, to

further our understanding of the evolution of an ancient and important part of the innate

immune system, the complement system.
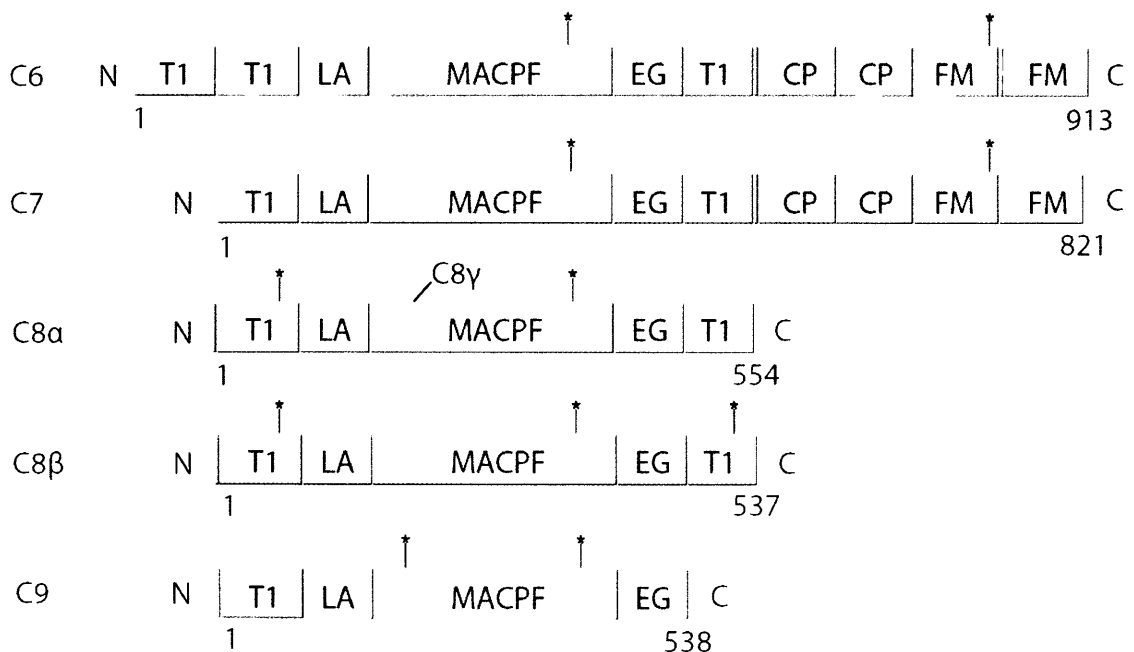
Figure 1. Modular structure of mammalian MAC protein family. Modules are labeled as follows: thrombospondin type I (T1), low-density lipoprotein receptor class A (LA), MAC-perforin (MACPF) domain, epidermal growth factor precursor (EG), Complement control protein (CP), Factor I module (FI). Numbers correspond to protein amino acid residues. Asterisks correspond to potential N-linked glycosylation sites.

CHAPTER II.

BACKGROUND

The nurse shark, *Ginglymostoma cirratum*, is a primitive member of the vertebrate phyla and, by virtue of its phylogenetic position, serves as an excellent animal model to study ancestral complement genes and proteins, including those of the MAC. The shark immune system has elements of innate and adaptive immunity and, for the purposes of this study, a functional complement system complete with a terminal lytic pathway (Jensen et al., 1973; 1981; Smith, 1998; Smith and Jensen, 1986). Hemolytic activity of shark serum has been known for decades (Legler and Evans, 1967), and trans-membrane pore structures formed on target membranes have been shown to be structurally similar to those formed by mammalian MAC (Humphrey and Dourmashkin, 1969; Jensen et al., 1981; Ramm et al, 1982). The shark is the earliest vertebrate for which complement-associated lytic activity has been definitively established and the functional homologues of shark C8 and C9 partially purified (Jensen et al., 1973; 1981); however, the complete molecular composition of shark MAC has not been determined, nor have genes and/or proteins of individual MAC components C6 through C9 been cloned or characterized. The mammalian complement system also includes a complex system of regulatory and control proteins some of which are cell surface-associated proteins (Liszewski et al., 1996).

Mammalian Complement

The complement system is an enzymatic cascade that was named after its ability to 'complement' antibodies in clearing pathogens from an organism (Atkinson and Frank, 1974a and b). Complement has been well-characterized in mammals and consists of three

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activation pathways that converge at C3 and ends in the formation of MAC via the terminal, lytic pathway, MAC (Muller-Eberhard, 1988; Götze and Müller-Eberhard, 1971).

Although the complement system is an integral part of innate immunity, it interacts with a component of the adaptive system and thus serves as a bridge between the two systems (Atkinson and Frank, 1974a and b). When complement is activated, a chain of sequential events ensue that leads to the generation of biologically active peptides and assembly of MAC on target membranes (i.e., bacterial cells and transformed self cells). This supramolecular complex penetrates target membranes to form doughnut-shaped holes and causes lysis of cells and organisms that leads to cell death (Podack, 1988).

Non-mammalian Complement

Complement has been present in a variety of diverse forms in taxa throughout evolution. Genetic evidence for a C3-homologue has been reported in coral, *Swiftia exertia*, (Dishaw et al., 2005) and halocynthins (Gross et al., 1999; Marino et al. 2002). One important element of complement conspicuously missing from organisms more basal than elasmobranchs is a terminal lytic pathway. Functional studies in the lamprey (a cyclostome that precedes the shark in evolution) have shown that the opsonic factors of the complement system are present, i.e., C3-Like proteins, but no evidence of assembly of a functional membrane attack complex has been detected (Nonaka et al., 1984), indicating that initiation pathways may be present, but terminated without assembly and execution of membrane attack. In the nurse shark, terminal lysis (Jensen et al. 1973;1981; Smith and Jensen, 1986) is present along with functional evidence of the

classical and alternative pathways (Smith, 1998), designating the shark as the most basal

organism to have a complete lytic complement system, similar to that found in mammals

and other higher vertebrates.

Mammalian MAC

MAC formation is initiated by C5b generated by the activation cleavage of C5

and followed by the sequential assembly of complement proteins C6, C7, C8 (α, β, and γ

subunits) and several molecules of C9. The insertion of C5b-C9(n) complex into the

target membrane disrupts membrane integrity by forming transmembrane pore-like

structures that make cells leaky (Muller-Eberhard, 1988) (Figure 2). The assembly of

MAC and its subsequent insertion into the lipid bilayer of target membranes depend on

the ability of the terminal components (C6 through C9) to undergo conformational

changes involving hydrophilic-amphiphilic molecular transition to expose hydrophobic

domains to the complementary binding domain of the succeeding MAC protein (Kolb et

al, 1972; Kolb and Muller-Eberhart, 1972; Sodetz, 1989; Hadders etal, 2007). A

conformational change in C5b allows the non-covalent attachment of C6. Binding of C7

to the C5b6 complex exposes concealed hydrophobic sites within the C7 molecule, thus

permitting insertion of the C5b–C7 complex into the target membrane. Anchored in this

way, C5b-7 serves as a receptor for C8. The C5b–C8 complex then undergoes a

conformational change that exposes a site on C8β that begins the polymerization of

multiple molecules of C9 (1–18 in humans) to form a barrel-like tubular structure through

the target membrane (Kolb and Muller-Eberhard, 1974; Podack et al, 1978). These

channels disrupt target membranes, leading to cell lysis and death from leakage (Podack

and Tschopp, 1984). The two terminal components, C8 and C9, are crucial for target cell lysis.
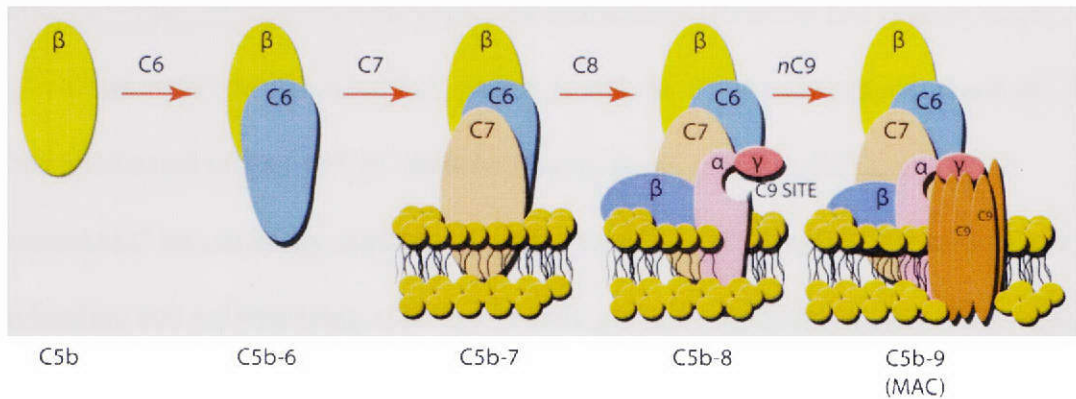
Figure 2. Sequential assembly of the membrane attack complex via the terminal lytic pathway of mammalian complement.

The lytic proteins of cytotoxic lymphocytes, natural killer (NK) cells, and MAC proteins share several common structural motifs, specifically the MACPF domain.   The damage caused by MAC is similar to the trans-membrane channels formed by perforin molecules of NK cells on target membranes (Young et al., 1986). Perforin is a protein found in cytolytic T and NK cells that polymerizes to form the same type of doughnut-shaped holes as MAC. In the presence of calcium, perforin polymerizes into transmembrane tubules and is capable of lysing, non-specifically, a variety of target cells (Podack et al., 1989).   It is generally believed that MAC proteins, C6 through C9 and perforin, arose through a series of gene duplications of an ancestral perforin-like gene

(Mondragon-Palomino et al., 1999). Thus the MAC complement proteins are considered members of a gene family that includes the perforins.

MAC proteins have high homology to each other, and their interactions are highly specific as is their interaction with target cell membranes (Brannen and Sodetz, 2007). The disulfide bond between C8α and γ subunits and the non-covalent binding site of C8β to C8α are located in the MACPF domain (Plumb et al., 1999). CD59 (protectin) prevents MAC formation by sequestering part of the MACPF domain of C8α to keep C9 from binding and polymerizing. CD59, a 20 kDa glycophosphatidylinositol-linked glycol protein that acts by binding to C8 and C9 to inhibit the formation of the lytic pore, is widely expressed, as it is crucial in preventing destruction of "self" cells by preventing MAC deposition (Kimberley et al., 2007). C9 requires the C8α LA module as well as the MACPF domain to complete MAC formation (Scibeck et al., 2002).

Two components of MAC differ in genetic structure and belong to non-MAC protein families: C5b and C8γ. C5 is cleaved by C5 convertase into C5α and C5b. C5α is an anaphlytoxin involved in the inflammatory response and causes smooth muscle contraction and vasodilation (Sodetz and Plumb, 2001). C5b is a cleavage product of activated C5 that is structurally similar and genetically related to the thioester-containing α₂Macroglobulin (a2M) protein family, which consists of C3, C4, C5, and a2M (Sodetz and Plumb, 2001), and differs from other MAC proteins quite distinctly in that it can non-covalently attach to target membranes to initiate the assembly of MAC, while C8γ is a lipocalin and modulates inflammatory responses (Haefliger et al., 1991). Parker and Sodetz (2002) have shown that MAC can be assembled without C8γ (Brickner and

Sodetz, 1984) . In addition, Trojer et al. (1999) have detected it in the human fetal and adult kidney independent of its C8γ counterpart, signifying that these subunits may have roles distinct from complement action. Furthermore, the C8γ gene (C8G) is located on a different human chromosome (9q) than C8α and C8β, distantly from C8A and C8B genes (human chromosome 1p32), and because it may not have undergone the gene duplication events it may have evolved separately from other MAC constituents (Platterborze et al, 1996: Rittner et al., 1986).

Duplication of MAC Genes

Biological systems contain many examples of genes that have arisen through the mechanism of gene duplication, which complementologists generally accept as playing an important role in the evolution of the complement system (Nonaka, 2001). Gene duplication in the complement system can be a unique strategy for the immune system to increase the range of recognition of foreign molecules (Sunyer et al., 1998; Nakao et al, 2006). The complement system consists of several examples of related proteins that have arisen by gene duplication, as shown by gene sequence similarity and repetitive modular domain analysis (Figure 1). Several sets of complement components have been discovered in the mammalian complement system that are believed to have arisen from a common ancestor: C1r/C1s, MASP1/MASP2, factor B/C2, C3/C4/C5, and C8α/C8β. These homologous proteins and genes indicate that gene duplication events are common in this system and have given rise to multiple components with similar structures, functions, and domains which are believed to strengthen the functional repertoire of the complement system (Nonaka and Smith, 2000). The overall component structure of complement of teleost and cartilaginous fish is similar to that of mammals; this suggests

that tetraploidization may have caused the increase in the number of components and emerged after divergence of the cartilaginous fish (Nonaka and Smith, 2000). In-depth sequence and domain analysis indicate that the MAC proteins C6, C7, C8α, C8β, and C9 underwent frequent gene duplication and exon-shuffling that resulted in sequential addition and deletion of the different modular domains (Volankis and Frank, 1998).

Module Similarity

Modules are protein scaffolds that have been conserved through biological evolution (Campbell, 2003). One purpose of modules is to provide a binding surface to facilitate interactions among a diverse array of macromolecules, like those of the MAC family, to form dynamic complexes (Campbell, 2003). The MAC proteins C6, C7, C8α, C8β, and C9 consist of several cysteine-rich domains or modules such as T1, LA, MACPF, and EG modules (Figure 1) that are commonly found in immune proteins, but that are more structurally and genetically related to each other than to other complement proteins (Sodetz and Plumb, 2001). Some of these modules were first described in systems that were non-complement and non-immune system-related proteins (Morley and Walport, 2000). One essential module present in all MAC proteins (with the exception of C5b and C8γ) is the MACPF domain (Figure 1). The presence of common modules is a primary element that suggests that these proteins arose from gene duplication.

Non-Mammalian MAC

Information on the composition of MAC for non-mammalian species is incomplete, particularly for lower vertebrates, although genes homologous to mammalian MAC genes and/or proteins have been described for birds (Mikrou and Zarkadis, 2010), frogs (McLin et al., 2008), and teleosts (Nakao et al. 1998; Papanastasiou and Zakardis,

2005). Little is known of the biological activities associated with these genes and how they compare with those of higher vertebrates (Scapigliati et al., 2001). Molecular tools have revealed complement genes in organisms as ancient as corals (Dishaw et al., 2005) and ascidians (Nonaka et al., 1999), demonstrating that complement-like activity is an ancient immune mechanism. However, a fully functional enzymatic cascade has been described only for the gnathostomes and other vertebrate species, and a functional terminal pathway has not been described in organisms more basal than the shark. Although genes and some components of the classical and alternative activation pathways have been found in agnathans, echinoderms (Smith et al., 1999; Smith et al, 2001) and even cnidarians that evolved prior to the protostome/deuterostome split (Dishaw et al., 2005), no associated target cell lysis has been observed in the afore named organisms and no MAC proteins have been isolated or genes cloned, with the exception of a C6-like gene that has been cloned from amphioxus, a chephalochordate (Suzuki, 2002). Whether the encoded protein functions as a component of a MAC in amphioxus remains to be determined. These genes suggest potential modular structures but are significantly different in composition (Suzuki, 2002).

Individual components analogous to those of the lytic pathway in mammals (i.e., C6 through C9) have been described only for few non-mammalian species. C8β has been cloned and characterized in the Japanese flounder, *Paralicththys olivaceous* (Kalgiri et al., 1999). All three C8 gene subunits have been isolated from carp, *Cyprinus carpio* (Uemura et al., 1996). C8 α, β, and γ genes have been cloned and sequenced in the rainbow trout, *Oncorhynchus mykiss* (Papanastasiou and Zarkadis, 2005; Kazantzi et al., 2003) and in the frog (*Xenopus tropicalis*) (genbank sequences, unpublished). Studies of

these genes show that the modular architecture (T1, LA, EG, MACPF) shown in Figure 1 has been conserved in amphibian and teleost MAC counterparts.

Shark MAC

Studies of the complement system in cartilaginous fish have shown functional parallels to mammals that share analogous components (Smith, 1998). Hemolytic activity of shark serum has been recognized for decades, and trans-membrane pore structures formed on target membranes have been shown to be structurally similar to those formed by mammalian MAC (Jensen et al., 1973:1981). The shark complement system has been shown to be structurally and functionally similar to that of humans and other vertebrates (Smith and Jensen, 1986; Shin et al., 2007; Graham et al., 2009; Shin et al., 2009). In the shark, C8 and C9 functional analogues can lyse target cells with human EAC1-C7 or EAC1-C8 cells, respectively (Jensen et al., 1973;1981). Although the shark is the earliest vertebrate for which complement-associated lytic activity has been definitively established, the molecular composition of shark MAC has not been determined nor have genes and/or proteins of individual MAC components C6 through C9 been cloned or characterized. Earlier functional data indicated the presence of C8 and C9 homologues (referred to as C8n and C9n or t1 and t2) in the shark and the proteins were functionally purified from serum.

Mammalian C8

Mammalian C8 is a trimeric oligomer composed of non-identical subunits, α, β, and γ chains, each encoded by a separate gene (C8A, C8B, and C8G) (Steckel et al., 1980; Ng et al, 1987). As a native composite serum protein, human C8 has a molecular weight of 151 kDa, composed of an α chain (64 kDa), a β chain (64 kDa), and a γ chain

(22 kDa) (Kolb and Muller-Eberhard, 1976). Structurally, the γ subunit belongs to the lipocalin family and is unrelated to the MAC complement protein family. C8A and C8B are located on chromosome 1p32, and C8G is located on 9q (Rittner et al., 1986).

While C5b-7 complex is assembled on the membrane, the binding of C8 to the complex embeds the complex further into the membrane, causing the target cells to slightly leak. C8 is the first MAC protein to completely insert in the lipid bilayer of the "target" membrane, initiating the anchoring of MAC, which is facilitated through the C8α subunit (Steckel et al., 1980). C8β in turn is non-covalently bound to the disulfide-linked C8α and C8γ subunits. The C8α subunit has a crucial role in completing the assembly of MAC since it rapidly binds and initiates the self-polymerization of C9 molecules which insert into the lipid bilayer and cause osmotic lysis (Musingarimi et al, 2002). C8α is a subunit that also contains a host of several essential binding sites for the formation and control of MAC function (Plumb et al 1999). It is a unique member of MAC since it is the only MAC protein that hosts a characteristic indel (insertion/deletion sequence) (Plumb and Sodetz, 2000).

Shark C8

Previous studies on shark have shown the presence of a 185 kDa C8 functional analogue (Jensen et al.1981); however, details of its primary, secondary, and subunit structure were unknown. The goal of the present study was to define shark C8 structure by characterizing the gene(s) of one or more C8 subunits. This report documents, characterizes, and presents the first nucleotide sequence of a MAC protein in the shark in an evolutionary context using phylogenetic analysis.

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CHAPTER III.

MATERIALS AND METHODS

Harvesting of Nurse Shark Tissues

A 2- Kg young female nurse shark was captured from the waters near the Keys Marine Laboratory (KML), Long Key, Florida, and transported in seawater to Florida International University (FIU) for sacrifice and subsequent tissue harvesting. The animal was anesthetized with 1 part per million (ppm) of 3-aminobenzoic acid ethyl ester (methane sulfonate) and allowed to bleed out from the caudal vein. After careful dissection, the tissues were flash-frozen in liquid nitrogen and stored at -80°C until used for nucleic acid extraction.

Shark Blood Collection and Processing

Captive adult nurse sharks were kept in an open seawater channel at the KML. All animals were tagged with an identification number to ensure that animals were not bled more frequently than once every eight weeks. Sharks were anesthetized by hand-capturing by net and placing into a concrete bath filled with seawater containing 1 ppm of 3-aminobenzoic acid ethyl ester (methane sulfonate). Anesthetized sharks were removed from the tank and placed in a supine position. Peripheral blood was aseptically drawn from the caudal vein with an 18-gauge sterile needle directly into a 30- or 60-ml syringe. After bleeding, the anesthetized sharks were returned to the water channel and walked to allow the water to flow through the gills until normal respiratory function was restored.

For shark DNA isolation from blood cells, shark blood was added to queen's lysis buffer in a 1:40 ratio and mixed by inversion for 10 m, then placed on ice in a dark cooler. To collect shark serum, shark blood was immediately placed in sterile 50-mL

polypropylene tubes, wrapped in foil, and placed on ice for transport back to the lab. The tubes were transferred to a 4°C refrigerator for 24 h to clot. Before centrifugation, a sterile wooden applicator stick was used to release the clot from the perimeter of the tube. The tubes were centrifuged in a Beckman GPR centrifuge at 4°C, 12,000 x g for 10-15 m. Separated serum was decanted in one fluid movement from the clot into a 50-mL sterile Pyrex glass tube without disturbing the clot. Since some erythrocytes and leukocytes do transfer with the serum, the serum was centrifuged again to remove all erythrocytes and cellular debris at 15,000 x g at 4°C for 15-20 m. Clear serum was decanted in to a 50-mL sterile glass Pyrex screw cap tube and stored in a circulating refrigerated water bath (NESLAB) at 0°C until further use for functional studies or Western blot analysis. For long term storage, serum was stored frozen at -20°C.

Total RNA Extraction from Shark Tissues

Shark tissues (liver, kidney, brain, intestine, ovary, muscle, heart, pancreas, spleen, erythrocytes, and leukocytes) were removed from the -80°C freezer and from each tissue a small sample of approximately 50-100 mg (or $1 x 10^7$ cells) was removed and placed into a 1.5 ml eppendorf tube and homogenized by Kontes Pellet Pestle® motor in 1-ml of TRIzol Reagent (Invitrogen Life Technologies) and left to incubate at room temperature for 5 m. Next, 200 ul of chloroform was added to each tissue sample homogenate, shaken vigorously, and incubated at room temperature for 2 m. Samples were centrifuged at 12,000 x g for 15 m at 4°C. After centrifugation, the RNA-containing aqueous phase was transferred to a sterile 1.5 ml eppendorf tube. RNA was precipitated by adding 0.5 ml isopropanol and the tube gently inverted and incubated for 10 m at room temperature. This mixture was centrifuged at 12,000 x g for 15 m at 4°C.

The supernatant was decanted, and the RNA pellet washed with 1 ml of 75% ethanol, vortexed, and spun at 7,500 x g for 5 m at 4°C. The ethanol was decanted and the RNA pellet left to air-dry for 10 m. The extracted RNA was dissolved in DEPC treated water and stored at -80°C.

First-strand cDNA Synthesis, Degenerate RT-PCR, and Product Cloning

Total RNA was extracted from homogenized nurse shark liver using the TRIzol Reagent (Invitrogen Life Technologies) according to manufacturer instructions (detailed above). Using Superscript II reverse transcriptase and Oligo(dT)12-18 primer (Invitrogen Life Technologies), first-stranded cDNA was synthesized using 4 ug of total RNA as the template. Degenerate primer NSC9-DGF1 (Table 1) was based on a highly conserved region (CNGDQDC, human amino acids 115-121) of human C6, C7, C8α, C8β, and C9-deduced amino acid sequences and employed in RT-PCR. NSC9-DGF1 was paired with abridged universal amplification primer (AUAP) (Clontech) for RT-PCR.

Table 1: Primers used for sequence analysis, synthesizing PCR- Digoxigenin (DIG) probes, and RT-PCR analysis of the GcC8α gene.

Primer Name	Sequence 5'- 3'	Location in sequence[a]
NSC9-DGF1	GYAAYGGNGAYAAYGAYTGYG	115–121
C8ASAZNFP3	CAAACAGCGAACACGAAGC	1712–1731
C8ASAZNRP1	GAAATCAACAAAGAACACAGAG	1916–1937
NSC8A-L2	GCCGAAAAATCCGAAGTGTA	143–162
NSC8A-L3	GACTGGAGGGAACTGCGATA	610–629
C8A33F	AGGCATTGGCACAGTCAG	1121–1138
C8AFP10	TGTCTGCCTGGTTATGAAGG	1606–1625
C8A-seqRP1	CTGGACTTTCTTGCTTCAC	218–236
C8A-SQRP1	TGGTTTTCGGTAGCATTTCTC	667–687
C8A-SQRP2	TTACCGAGCCACCCACA	1212–1228
C8A-SQRP3	GGCACGCTTTCCCTTCAT	1619–1636

C8a-ESFP2	ATTACACTGCATGAAGAATGA	11-31
C8a-ESRP1	CTGGTAATGATGGACCTGG	2075–2093
beta-actin F	CTGCCATGTATGTTGCCATC	389-408
beta-actin R	ATCCACATCTGCTGGAAGGT	1051-1070
M13F	TGTAAAACGACGGCCAGT	
M13R	GTTTTCCCAGTCACGAC	

[a] Indicates the amino acid position in human C8α chain

Conditions for Amplification of GcC8α DNA sequence

The thermocycler was set for 94°C for 1 m and 35 cycles of 94°C for 30 s, 56°C

for 30 s, and 71°C for 3 m and for a final extension for 6 m. DNA fragments of 2.1 and

2.9 Kb were detected by electrophoresis and further amplified by nested PCR

(SMARTRACE cDNA amplification kit, Invitrogen Life Technologies) under

thermocycler settings of 94°C for 30 s, 62°C for 30 s, 72°C for 3 m, and 30 s for 25

cycles. The PCR products were run on a 1% agarose gel and further purified using the

QIAquick gel extraction kit (Qiagen) according to manufacturer's instructions. The

extracted products were cloned into a TOPO-TA 10F' vector (Invitrogen Life

Technologies). Recombinants were identified by blue/white colony selection on

ampicillin-containing LB agar plates.

Preparation of Plasmid DNA and Sequencing

Clones positive for the expected 2.1 Kb insert (based on primer design) were

selected by colony PCR. Colony PCR amplification was carried out for 30 cycles of 94°C

for 30 s, 51°C for 30 s, and 72°C for 2 m. Plasmids of selected clones were purified

using the SV Minipreps DNA purification system (Promega). The purified plasmids

were subjected to cycle sequencing reactions composed of 2μl Big Dye Terminator V3.1

(Applied Biosystems); 4 μl purified plasmid DNA (35 ng/μl); 2 μl Bigdye terminator

buffer; and 2 µl (0.8 µM) gene-specific primer. All cycle sequencing reactions were carried out as follows: initial denaturation at 96°C for 1 m, followed by 30 cycles of 96°C for 5 s, 50°C for 10 s, and 60°C for 4 m (Sanger et al., 1977). The resulting cycle sequencing products were submitted for sequencing by the ABI377 (Applied Biosystems) automated sequencer at the FIU DNA Sequencing Core facility. Clones were subjected to cycle sequencing using M13 forward and reverse primers (Table 1); gene specific primers were constructed from resulting sequences to further sequence the entire gene. All sequencing primers were designed to overlap at the 3' end of each reading frame by at least 100 base pairs (Figure 3). Gene specific primers used to identify and sequence GcC8α gene are listed in Table 1.
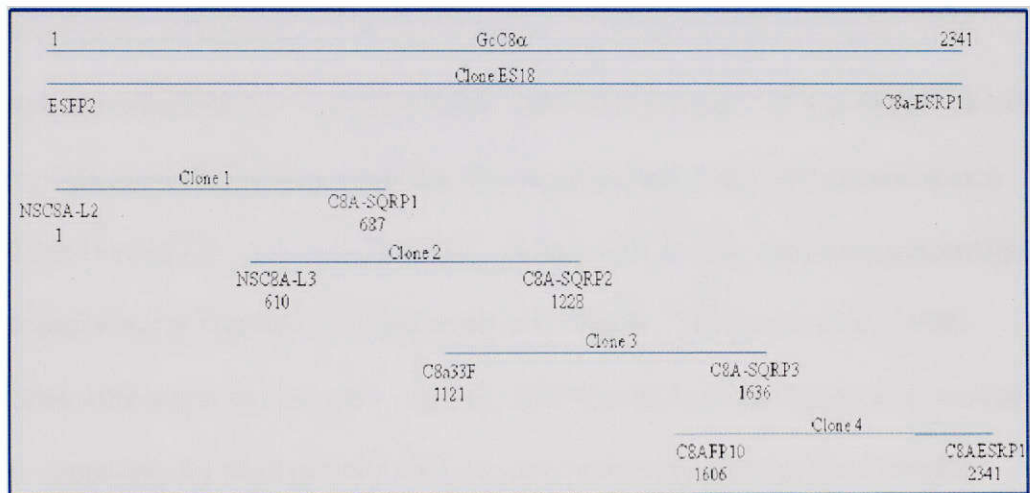
Figure 3: Schematic of primer local and overlapping clones of GcC8α gene.

Amplification and Cloning of Full-Length GcC8α

A full-length GcC8α transcript was obtained by long-PCR using primers C8a-ESFP2 and C8a-ESRP1 that were designed to the 5' and 3' UTR of the assembled

sequence generated from overlapping clones (Figure 3). Amplification was carried out for 38 cycles: 94°C for 30 s, 60°C for 30 s, and 70°C for 3 m. The PCR mixture was composed of 1 µl of each primer (10 µM), 45 µl PCR Supermix High Fidelity (Invitrogen Life Technologies), and 3 µl cDNA. The PCR product was run on a 1% agarose gel with ethidium bromide. The band of expected size was cut out, gel-purified, cloned, and sequenced as described in the cloning procedure employed for the preparation of plasmid DNA and outlined above. Clones positive for the 2.5 kb insert were selected by colony PCR.

Sequence Compilation and Phylogenetic analysis

The full-length GcC8α nucleotide sequence was assembled from over-lapping clones (Figure 3) and translated to the corresponding amino acid sequence in the BioEdit biological sequence alignment editor for Windows 95/98/NT/2000/XP (Hall, 1999). Using gene specific primers a complete full-length GcC8α transcript was identified. The identities of positive clones were established using the Basic Local Alignment Search Tool (BLAST) search engine (Altschul et al., 1990). Identity and similarity percentages were calculated using alignments constructed in ClustalW (Thompson et al., 1994). Calculations were made by manually counting identical and similar amino acid residues. Multiple alignments for phylogenetic analysis were constructed using the ClustalX program (Thompson et al., 1997). This alignment was then used in the PAUP* program (Phylogenetic Analysis Using Parsimony, Swafford DL, 2002) to construct a phylogeny using the neighbor joining algorithm (Saitou and Nei, 1987) under the default settings. Confidence in the branch points was validated by 1000 bootstrap replications. Sequences for other species were obtained from GenBank.

Molecular Analyses

Molecular modules were determined by studying and comparing alignments created by ClustalW of GcC8α sequence and C8α sequences of other taxa. Potential N-linked glycosylation sites were predicted by the presence of the amino acid sequon N-X-[S or T] (Marshall, 1974), where X is any amino acid, followed by a Serine (S) or Threonine (T) residue (Figure 4). Potential C-mannosylation sites were identified by searching for the sequon W-X-X-W-X-X-W, where X is any amino acid (Hofsteenge et al., 1999). Hydrophobicity profiles were generated under the default settings of the Kyte & Doolittle (1982) algorithm in the BioEdit program (Hall, 1999).

Southern Blot Analysis

Southern blot analysis was employed to determine GcC8α gene copy number. Southern blots were developed using a nylon membrane (Hybond N^+, currently GE Healthcare) that had previously been probed by a DIG labeled probe for another shark complement gene. The following describes how the membrane for the first probe was constructed, how this initial DIG-probe was removed, and the membrane was further hybridized with a probe designed specifically for GcC8α.

DIG-probe synthesis

DIG-labeled probe was synthesized using the PCR DIG Probe Synthesis Kit (Roche) to detect copies of the GcC8α gene in restriction enzyme digests of shark genomic DNA using the enzymes *Bam*HI, *Eco*RI, *Hind*III, and *Pst*I. Using the pattern of human C8α intron/exon as a template, the primer set C8ASAZNFP3 and C8ASAZNRP1 was designed to cover a 226 nucleotide sequence that did not extend across C8α introns

(Morley and Walport, 2000). A plasmid (4 ng/µl) containing the full-length GcC8α

sequence was used as the template for PCR reaction. Amplification consisted of 35

cycles of 95°C for 30 s, 54°C for 30 s, and 70°C for 30 s.

Table 2: DIG-Labeled probe PCR reaction mixture. All buffers, stock, and enzymes
provided by PCR DIG Probe Synthesis Kit (Roche).

Reagent	DIG-Probe	Unlabelled Control
Autoclaved water	16.625 ul	16.625 ul
PCR Buffer	2.5 ul	2.5 ul
PCR DIG label mix	2.5 ul	None
dNTP stock	None	2.5 ul
Forward Primer	1 ul 10 uM	1 ul 10 uM
Reverse Primer	1 ul 10 uM	1 ul 10 uM
Enzyme Mix	0.0375 ul	0.0375 ul
Template DNA	1 ul of 4 ng/ul	1 ul of 4 ng/ul
Total	25 ul	25 u

Restriction Enzyme Digestion

Shark genomic DNA (711 ng/µl) was digested with restriction enzymes *Bam*HI,

*Eco*RI, *Hind*III and *Pst*I at 37°C for 21 h. The restriction enzyme digestion mixture

consisted of 300 µl of 0.2 X TE Buffer (Invitrogen Life Technologies); 37.5 µl RE Buffer

3 (Invitrogen Life Technologies); 30 µl (711 ng/µl) shark genomic DNA; and 8 µl (10

units/µl) restriction enzyme (Invitrogen Life Technologies).

Ethanol Precipitation

Post-digestion, the nucleic acid was subjected to ethanol precipitation by adding

625 ul of 100% ethanol to each tube. The tubes were vortexed and incubated at room

temperature for 10 m. The digested DNA was further centrifuged at 3250 x g at 4°C for

20 m. The supernatant was then discarded and 1.2 ml of 70% ethanol added and vortexed.

This mixture was centrifuged at 3250 x g for 5 m at 4°C and the supernatant discarded. The tubes were then inverted on clean tissue paper for 20-27 m to air-dry the DNA. Finally, the precipitated DNA was re-suspended in 80 μl of 0.2X TE buffer and stored at 4°C until Southern Blot analysis was performed.

Electrophoresis of Digested Shark Genomic DNA

The digested shark genomic DNA (5 μg/lane) was electrophoresed in 0.8% agarose gel at 22 V for 10 h. The gel was then stained in 300 ml (0.5 μg/ml) of ethidium bromide in 1X TAE buffer. The digested DNA was further cut by immersing and agitating the stained gel in 500 ml of 0.25 N HCl at room temperature for 20 m. The DNA on the gel was denatured to keep the DNA fragments single stranded in 0.2 M NaOH/0.6 M NaCl for 15 m at room temperature twice. The gel was then rinsed with deionized water. The DNA from the gel was transferred onto a Hybond N+ nitrocellulose membrane by capillary action blotting for 17 h at room temperature. The DNA was immobilized under the "optimal" (120 mJ) setting option of the UV cross-linker.

Reprobing Southern Blot

As stated earlier, a previously probed membrane was used after stripping the membrane. To remove existing probe the used membrane was rehydrated in ddH$_2$O for 2-3 m, then submerged in 0.2 N NaOH/0.1% SDS probe removal solution under agitation at 37°C for 10 m. The membrane was removed from the solution, washed a second time with the probe removal solution, and incubated a second time. The membrane was then equilibrated by agitation in 2X SSC at room temperature for 2-3 m. The membrane was placed in a hybridization tube with 5 ml of 42°C prehybridization solution (5XSSC, 2% milk, 7% SDS, 0.05 mg/ml salmon sperm DNA in ddH$_2$O) and incubated in a

hybridization chamber at 42°C for 2-4 h. After prehybridization, hybridization solution (5 ml prehybridization solution and 10 μl DIG-labeled probe) was added to blot in the hybridization tube and incubated overnight (16-18 h) at 42°C. The membrane was removed from the hybridization tube, placed in a wash solution (2X SSC, 0.1% SDS in ddH$_2$O), and agitated for 15 m at 42°C. This wash step was repeated two times. Then, stringent washing was performed twice in 0.1xSSC and 0.1% SDS for 30 m at 42°C. Then, DIG buffer 3 was added to the membrane and agitated for 5 m at room temperature. The membrane was heat-sealed in a plastic bag with 1 ml LUMI-PHOS Plus solution (Lumigen), wrapped in foil, and incubated for 45 m at 37°C. Finally, under red light, x-ray film was placed along with the membrane in a film cassette for 3 h. After incubation, the film was removed and placed in developer solution (Kodak) until band formation was visualized. The x-ray film was dipped in stop solution (20% acetic acid in ddH$_2$O) for 2 m, and finally into fixer solution (Kodak) for 3 m. The film was washed by running water and then hung to air dry.

GcC8α Gene Expression Analysis by RT-PCR

As described, the first-strand cDNA that was synthesized from each tissue was used as a template for RT-PCR. Employing the primers NSC8A-L2 (forward) and C8A-SQRP1 (reverse) (Table 1) that span a 503 nucleotide region of the GcC8α sequence, RT-PCR was performed under thermocycler settings of 42 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 50 s. Universal β-actin specific primers for β-actin (forward: 5'-CTGCCATGTATGTTGCCATC -3' (nucleotide numbers, 389-408) and reverse 5'-ATCCACATCTGCTGGAAGGT-3' (nucleotide numbers 1051-1070) were run simultaneously as a control at the same thermocycler settings except that amplification

was carried out for 32 cycles. PCR products were electrophoretically analyzed on a 1%

agarose gel containing ethidium bromide.

Primer Resuspension

All primers were purchased desalted from Sigma- Genosys (currently Sigma-

Aldrich) and reconstituted to 100 μM stock solutions in autoclaved sterile ddH$_2$O. All

primers were stored at -20°C after resuspension.

SDS-PAGE

Preparation of Supernatant 1 from shark serum

Low ionic strength precipitation of shark serum was achieved by adding cold

water to reduce ionic strength of serum to 4 mS as measured by a conductivity probe.

The diluted serum was then kept on ice and swirled intermittently for 1 h. The diluted

serum was centrifuged at 1000 x g for 40 m to separate and remove the precipitated

proteins (Precipitate 1) from the supernatant (Supernatant 1), which was stored at 4°C

until used in SDS-PAGE and Western blot procedures. Lowering the ionic strength of

shark serum by dilution results in precipitation of the bulk of proteins such as C1q and

shark immunoglobulin which can interfere with reactions in Western blots.

Sample Preparation

Samples of human serum, shark serum, and shark Supernatant 1 sample were

prepared for SDS-PAGE electrophoresis according to Table 3. Physiological saline was

used when samples were diluted as described in Table 3. Gels designated for Western

blot analysis were loaded with pre-stained molecular weight standards while gels that

were to be stained with Coomasie blue were loaded with unstained protein standards. All

samples and reference standards were heated to 95°C in a water bath for 5 m and

centrifuged at 8000 x g before loading; 25 µl of each sample was loaded onto a separate lane in the gel.

Table 3: SDS-PAGE sample preparation and lane allocation

Lanes	1	2	3	4	5	6	7
Sample	SS	SS-R	Spt1	Spt1-R	HS	HS-R	BRPS
Dilution	1:50	1:50	None	None	1:50	1:50	
Sample							
ul Serum/Spt1	1	1	20	20	0.66	0.66	
ul Physiological Saline	19	19	None	None	19.34	19.34	
ul R buffer		5		5		5	
ul NR Buffer	5		5		5		

NR: non-reduced, R: reduced, SS: shark serum, Spt1: Supernatant 1, HS: human serum, BRPS: Broad Range Protein Standards

Using Bio-Rad Criterion electrophoresis cell with Bio-Rad Model 200/2.0 power supply, gels were run in 1X tris-glycine running buffer at 200V until the dye front had migrated to the end of the gel. When the dye front reached the bottom of the gel, electrophoresis was halted and the gel assembly dismantled. The left side of the gels was nicked to show the direction in which the gel was loaded, the gels were then immersed in Fermentas PageBlue staining solution for 5 m and rinsed in water until most of the dye was removed. After destaining (20% methanol, 10% acetic acid), the gels were photographed, then wrapped in one layer of cellophane and vacuum-dried on a Model 543 gel dryer (Bio-Rad).

Western Blot Protocol

Four pairs of SDS-PAGE gels were run as described above. One gel of each pair was used to stain as described to visualize all protein bands of each sample, while the

other was used to blot. The blotting gels were rinsed in water and assembled into the blotting sandwich. The sandwich was assembled as follows: the cassette was laid black side down. A fiber pad was placed on the black side, then filter paper, the gel, the nitrocellulose membrane, more filter paper, and last, a fiber pad. The cassette was closed and placed in the transfer chamber. Prior to assembly, all sandwich materials (nitrocellulose membrane, filter pads, and filter paper) were equilibrated in Nupage transfer buffer (Invitrogen Life Technologies). An ice block was added to the chamber along with a stir bar to prevent damage to the blot from the heat generated by the electrical current. The chamber was filled with transfer buffer, placed on a stir plate, and connected to the power supply. The apparatus was subjected to 100 V for 1 h. To confirm complete protein transfer from gel to membrane, the sandwich was disassembled and the gels were stained with Coomasie blue which would stain proteins that did not transfer and remained in the gel. All washing and incubations were subjected to agitation to ensure adequate homogenous covering of the membrane and prevent uneven binding. The membranes were immersed in blocking solution of 5% milk (Carnation) for 1.5 h in a dark container. The solution was replaced with fresh blocking solution and the blots were left at 10 °C overnight. The blocking solution was removed and wash diluent buffer (WDB: 200 ml 1XTBS, 2 g instant milk, 100 µl Tween 20) was added to the membrane and agitated for 10 m. This step was repeated three times. Then, 10 ml of primary antibody solution (1XTBS, 1% milk, and 1:200 diluted goat anti-human C6, C7, C8, or C9 antibodies purchased from Quaigen) was added and agitated for 3 h. The blot was then washed with WDB for 20 m twice at room temperature, then 10 ml of secondary antibody solution (1XTBS, 1% milk, and peroxidase-tagged rabbit anti-goat

immunoglobulin (Ig) secondary antibody (1:500)) was added to the blot and incubated for 1 h with agitation. The secondary antibody solution was decanted and the blot was washed twice with WDB for 10 m each time. The protein blot was then developed by the addition of BioRAD HRP (horse radish peroxidase) Conjugate Substrate kit until reactive bands appeared. Precaution was taken not to allow development to proceed for too long in order to avoid and minimize non-specific reactions.

CHAPTER IV.

RESULTS

Cloning and Sequence Analysis of the Full-Length GcC8α cDNA

A 2341 nucleotide sequence was constructed from overlapping clones (Figure 3 and 4)

that included the 3' and 5' UTRs. Table 1 lists the primers used. From these overlapping

clones a single cDNA sequence was determined (Figure 3 and 4). Gene specific primers

designed C8a-ESFP2 and C8a-ESRP1, were designed based on the compiled sequence

and used in PCR amplification (Frohman et al., 1988) to generate a mRNA transcript

representing the full-length shark C8α gene. Several clones representing a single full-

length cDNA sequence with the 3' and 5' UTRs and high homology to C8α of the

human, mouse, rat, rabbit, and pig were identified. The nucleotides (1770) of the coding

region were translated using the BioEdit program (Figure 4) into 589 amino acid

residues.

GcC8α Percent Identity and Similarity Across Taxa

Using the computer software ClustalW, the deduced GcC8α amino acid sequence

was aligned with other known C8α amino acid sequences of the human, mouse, rat, pig,

and rabbit (Figure 6). The percentage of amino acid identity and similarity between

GcC8α and C8α sequences from other species was calculated (Table 4). The average

amino acid sequence identity between the nurse shark C8α gene and human, mouse, rat,

pig, and rabbit known C8α sequences ranged from 38.7 – 47.4% identity and 65.4 –

78.4% similarity.

```
1    AAAAAATATTATTACACTGCATGAAGAATGAATATGTTTTTGAGCTGCTTCTCAACTTTTGCATGCCTGCTGTTTTTTACTCATTTTGCC 90
                                   M  F  L  S  C  F  S  T  F  A  C  L  L  F  F  T  H  F  A

91   AATTCGAATCAGATCAGCAACAGTTCAAAACATTGTTGCACGCATCAGTCTGGCCGAAAAATCCGAAGTGTAGTCCCACTTGACTGTCAA 180
     N  S  N  Q  I  S  N  S  S  K  H  C  C  T  H  Q  S  G  R  K  I  R  S  V  V  P  L  D  C  Q

181  CTTGGCCAGTGGGCACAATGGACAGTGTGTTCTCCTTGTGAAGCAAGAAAGTCCAGATATAGGAATCTGAATCGGCCAGCCATATATGGT 270
     L  G  Q  W  A  Q  W  T  V  C  S  P  C  E  A  R  K  S  R  Y  R  N  L  N  R  P  A  I  Y  G

271  GGTAGCCAATGCATTGGATCTCTCTGGGAAGACACATCTTGTGAAACATCTGAACAATGCATACCAAAAATTAACTGTGGTGATCAGTTT 360
     G  S  Q  C  I  G  S  L  W  E  D  T  S  C  E  T  S  E  Q  C  I  P  K  I  N  C  G  D  Q  F

361  CAATGTAGTTCAGGTCGATGTATTAAAAGACATTTACTGTGCAATGGTGAGAAAGACTGTGCAGATGTTTCAGATGAAGAAACCTGTGAG 450
     Q  C  S  S  G  R  C  I  K  R  H  L  L  C  N  G  E  K  D  C  A  D  V  S  D  E  E  T  C  E

451  TCTGATTATCCATATGAGAGAAGGACTTTCTGCAGTGACTTGTTTTTAATCCCAGGCATAGAAGCAATTATGACTGGCTATAACATCTTA 540
     S  D  Y  P  Y  E  R  R  T  F  C  S  D  L  F  L  I  P  G  I  E  A  I  M  T  G  Y  N  I  L

541  ATACATGACGTTGGAAGAACTGTGTTAGACACTGGGTTTGGTGGATATTGTGAATATGTGTATAATGGGGACTGGAGGGAACTGCGATAT 630
     I  H  D  V  G  R  T  V  L  D  T  G  F  G  G  Y  C  E  Y  V  V  Y  N  G  D  W  R  E  L  R  Y

631  GACAGTGAGTGTGAACGCCTCTACTATAACGATGATGAGAAATACTACCGAAAACCATACAATTTACTCACATACCGTTTTGAGGCAATT 720
     D  S  E  C  E  R  L  Y  Y  N  D  D  E  K  Y  Y  R  K  P  Y  N  L  L  T  Y  R  F  E  A  I

721  GCTGATTCTGGATTTACCATAGATGTTCACAATGATGTACATGAATTAATTACAGCAATGAAGCATACAGACTCCTTTGATTTTGCAGTC 810
     A  D  S  G  F  T  I  D  V  H  N  D  V  H  E  L  I  T  A  M  K  H  T  D  S  F  D  F  A  V

811  GGTGTGCAAGTTTCTGTTGTAAAAGCTGCTATTGCTTTTGGAGAATATTCCACATTTATTAGAAATGTATCAAGGTTTCAAGGGAAGGAT 900
     G  V  Q  V  S  V  V  K  A  A  I  A  F  G  E  Y  S  T  F  I  R  N  V  S  R  F  Q  G  K  D

901  GTCAGTTTTGTTCGTGTTCGTACTAAGATTCAAACTGCTCACTTCAAAATGAGGAGATATAATTTACTTTTGGATGAAGATATGACTCAA 990
     V  S  F  V  R  V  R  T  K  I  Q  T  A  H  F  K  M  R  R  Y  N  L  L  L  D  E  D  M  T  Q

991  TCCCTCATGAAACTTCCAGATGAATACAATTACGGAATGTACGCTAAATTCATTGCAGATTATGGTACTCATTACTATGCTTCAGGAACA 1080
     S  L  M  K  L  P  D  E  Y  N  Y  G  M  Y  A  K  F  I  A  D  Y  G  T  H  Y  Y  A  S  G  T

1081 ATGGGAGGTGTTTATGAGTTCATTCTTGTTCTGAATAAGAAGGCATTGGCAGAGTCAGATTTAACTGCATCCGAAGCTGGATTTTGCGTT 1170
     M  G  G  V  Y  E  F  I  L  V  L  N  K  K  A  L  A  E  S  D  L  T  A  S  E  A  G  F  C  V

1171 GCAGGATCACTGGGAGTAGTGGTTTCAAAAGGAACCATGGATGTGGGTGGCTCGGTAAAGGCCAAAGGTTGTAGACGTAAATTGGAGCAG 1260
     A  G  S  L  G  V  V  V  S  K  G  T  M  D  V  G  G  S  V  K  A  K  G  C  R  R  K  L  E  Q

1261 AATGAACCTTCAGAAAAGTCAAGAAGCCTTGTTGAAGATGTTCTTCCACATGTACTGGGGAGGTGACCTGAAATCAAGTGCTGGACTATTA 1350
     N  E  P  S  E  K  S  R  S  L  V  E  D  V  L  P  H  V  L  G  G  D  L  K  S  S  A  G  L  L

1351 GGTCATGGAATACCAGATGTCAAAATGTATCGGCATTGGGGTAAATCTTTGAAATACCTGCCAGCTGTCATTGACTTTGAGCTCATGCCA 1440
     G  H  G  I  P  D  V  K  M  Y  R  H  W  G  K  S  L  K  Y  L  P  A  V  I  D  F  E  L  M  P

1441 ATTTATGACTTGGTGGCCAGAAGTAAACTTCAGTCTGTTGAAATCAAACAACAAAATTTAAAAAGAGCCATGGAAGAGTACTTGGTGGAG 1530
     I  Y  D  L  V  A  R  S  K  L  Q  S  V  E  I  K  Q  Q  N  L  K  R  A  M  E  E  Y  L  V  E

1531 TTTCATCCCTGTAGGTGTCCTGTGTGCTATAATAATGGAAAAGCAGTTCTTCTGGACAATGTCTGTACCTGTGAATGTCTGCCTGGTTAT 1620
     F  H  P  C  R  C  P  V  C  Y  N  N  G  K  A  V  L  L  D  N  V  C  T  C  E  C  L  P  G  Y

1621 GAAGGGAAAGCGTGCCAGGATACTAAACGAAAGGGGCCCACTAATGGAAATTGGAGCTGTTGGTCAGGGTGGTCTTCTTGCCAGAATGGT 1710
     E  G  K  A  C  Q  D  T  K  R  K  G  P  T  N  G  N  W  S  C  W  S  G  W  S  S  C  Q  N  G

1711 TCAAACAGCGAACACGAAGCTGTAACCATCCACCCCCTAAAGATGGTGGTGCTACATGCCTTGGGAAGAACACACAAACCAAGCATTGT 1800
     S  K  Q  R  T  R  S  C  N  H  P  P  P  K  D  G  G  A  T  C  L  G  K  N  T  Q  T  K  H  C

1801 TAAAACAAAATAATACCTCTGGCATTTTGGCCCGTTGAATTATTTGCTTTTGGGTGATCATTATTTTCTTTGAGTATAGATGAATATGAG 1890
     *

1891 GTATAGGCAACATTTTGAAATACTACTCTGTGTTCTTTGTTGATTTCTTAAATGTAATGAATGTTGAGATGTATTCATTGATGTGAAACA 1980
1981 TTTGAACTGACGTGACAAGAATTATCCTTTATTGAAGAAAGATTAAAACTGATTTGTGTGCATTAATGTTTGGCCAGGAAGCTTGTTCAC 2070
2071 ACTTCCAGGACCATCATTACCAGTTGATTGGTCAACATACTGCAATGAATTATGGGAGTCAGCATTGCAATAGACCTCATTGTGATGAAT 2160
2161 ACATGTTGACCTTGATATCTGATCCATTGTTTTCTTTACAGCAGGAACAGAATGGTAGTAAGAAAAATATGAAAATCAAATCAATATATT 2250
2251 AGCAGCATTAAATTCTGTACAGGATATCAAAGCAGTTATGATTCCTATTACTAATAAAGAGATGCCAAAAAAAAAAAAAAAAAAAAAAAAAA 2341
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Figure 4. Nucleotide and deduced amino acid sequence of GcC8α cDNA. The nucleotide sequence is above and the deduced amino acid sequence is below. Underlined bold letters indicate initiation codon, stop codon, and polyadenylation recognition signal, and the polyadenylation tail sequence. Putative N-linked glycosylation sites are indicated by bold, italicized N's and mannosylation sites indicated by bold, italicized sequences beginning and ending with W.

Table 4. Percent Identity and Similarity between the deduced amino acid GcC8α sequence and C8α homologues of other taxa

Organism	Identity	Similarity
Human	41.4	76.2
Rabbit	40.9	76.9
Chicken	36.9	65.4
Mouse	42.6	78.4
Rat	40.6	72.2
Pig	44.0	77.1
Frog	47.4	77.4
Rainbow Trout	38.7	70.1

Phylogenetic analysis

Multiple alignments for phylogenetic analysis were constructed by the ClustalX program (Thompson et al., 1997) using entire protein sequences of known MAC amino acid sequences, obtained from GenBank (Table 5) and GcC8α. This alignment was then used by the PAUP* program (Swafford, 2002) to construct a phylogeny using the neighbor joining algorithm (Saitou and Nei, 1987) under the default settings (Figure 5). Human, mouse, cow, cat and woodchuck perforin sequences were used as an out group. Confidence in the branch points were validated by 1000 bootstrap replications. GcC8α forms a clade with C8α sequences from other taxa and is sister taxa with *Xenopus* C8α. The tree also shows that the C8 complex has diverged from a common ancestor with C9.

Table 5. Accession numbers correlating with sequences used in Figure 6 phylogeny

Organism	Accession number	Organism	Accession number	Organism	Accession number
Human C6	BAD02321	Mouse C7	XP_356827	Rabbit C8α	AAA31191
Trout C6	CAF22026	Human C8α	NP_000553	Chicken C8α	XP_426667
Frog C6	AAH76972	Trout C8α	CAH65481	Xenopus C8α	AAH74554
Amphioxus C6	BAB47147	Chimpanzee C6	NP_001009015	Zebrafish C9	NP_001019606

Human C7	CAA60121	Dog C6-1	XP_868028	Human C8α	NP_000553
Trout C7-1	CAD92841	Dog C6-2	XP_536488	Human C8β	NP_000057
Trout C7-2	CAF22025	Rat C7	XP_226803	Cow C8β -1	XP_590870
Chicken C6	XP_429140	Chicken C7	XP_424774	Cow C8β -2	XP_870144
Xenopus C6	AAH76972	Flounder C7	BAA88899	Dog C8β	XP_536694
Zebrafish C6	NP_956932	Shark C8α	EF654112	Mouse C8β	NP_598643
Pig C7	NP_999447	Trout C8β	AAL16647	Flounder C8β	BAA86877
Human C9	NP_001728	Trout C9	P06682	Fugu C9	AAC60288
Rat C9	NP_476487	Mouse C9	NP_038513	Grass Carp C9	AAS76086
Trout C9	CAJ01692	Killifish C9	AAR87007	Flounder C9	BAA86878
Dog C9	XP_536494	Human perforin	AAA60065	Orangutan C6	BAD02323
Flounder perforin	BAC76420				
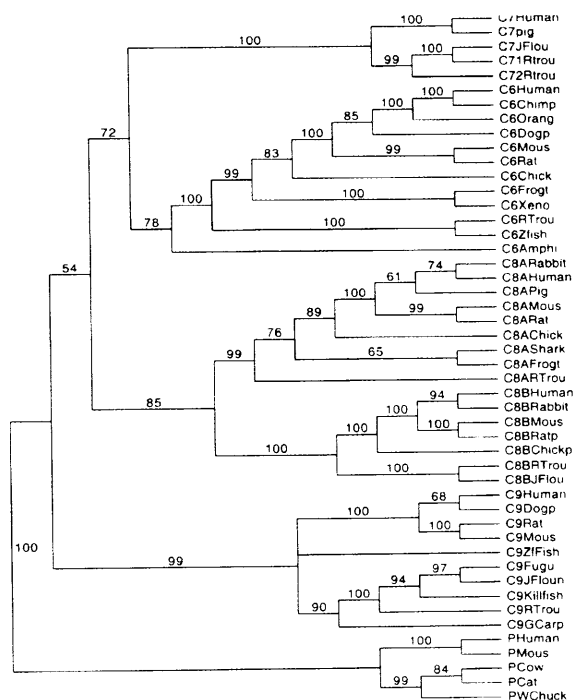
Figure 5. Phylogenetic analysis of MAC amino acid sequences across taxa. Alignment completed in ClustalX and phylogenetic tree generated by PAUP rooted on perforin from three taxa. Accession numbers of the sequences used to construct the tree are listed in the Table 5.

Multiple Alignment and Sequence Analyses

Using the computer software ClustalW, the shark C8α–deduced amino acid sequence was aligned with other C8α amino acid sequences of human, mouse, xenopus, and trout (Figure 6). The shark C8α sequence contains 33 cysteine residues of which 29 are conserved between the shark and human sequences. These residues are potentially capable of forming, through disulfide bonds, the characteristic C8α cysteine backbone suggesting a similar folding pattern and function to mammalian, amphibian, and teleost C8α. The GcC8α sequence includes the cysteine residue at 164 in human C8α and forms the disulfide bond with C8γ (Brickner and Sodetz, 1985). The nurse shark C8α sequence also conserves the cysteines that correspond to C^{324} and C^{349} in human that are proposed to form a disulfide bridge (Peitsch et al., 1990) (Figure 6). The multiple alignment also confirms the presence of the indel (human aa 159-175, shark aa 198–207) exclusive to C8α (Figure 6 and 7).

Analysis of the primary structure showed that GcC8α has a modular structure consistent with that of C8α of other taxa examined (Figure 8). The MAC-conserved modules identified in GcC8α are similar to those of mammalian species. The two T1 repeats, LA repeats, EG, and MACPF segments are present in GcC8α and are highly conserved (Figure 6 and 8).

Two potential N-linked glycosylation sites, at ASN^{26} (in the 5' UTR) and ASN^{281} (Figures 4 and 8), were identified in the sequence. The sequence was also examined for potential mannosylation sites; two were identified: one in the first T1 repeat in the pattern of WxxW amino acids 532-565 WAQW and the second site in the WxxWxxW motif and

```
                                                    Leader peptide
GcC8a       --------MFLSCFSTFACLLFFTHFANSNQISN--------------SSKHCCTHQSGRKJRSVVPLDCQLGQW   52
xenopusC8a  ---------------MIVMLYHALALWGYT---------------ASIGNMDLLLFRSIAAPQPEDCQLDQW        42
humanC8a    ----------------MFAVVFFILSLMTCQPG--------------VTAQEKVNQRVRRAATPAAVTCQLSNW     44
mouseC8a    ----------------MFVVAFFGLSLVAWHPG--------------VTAQEKVNQRVTRAVTPQAVSCQLSDW     44
RtroutC8a   MSSFSSRSSFISNMNRLICVLLGSYILLLVLNKSPTVDATEYSWNMAETRTGQSAIFRVRSVNKFAPINCKMKMW    74

                                TSP1                      LDLRA
GcC8a       AQWTVCSPCEAPKSRYRNLNRPAIYGGSQCIGSLWEDTSCETSEQCIPR-INCGDQFQCS-SGRCIKRHLLCNGE  126
xenopusC8a  SQWTSCFPCQQKKYRYRKLLQPAKYEGRPCVGSLWASMACQTAQKCVPE-NNCGNDFQCRDSGRCIKRPLVCNGD  116
humanC8a    SEWTDCFPCQDKKYRHRSLLQPNKFGGTICSGDIWDQASCSSSTTCVRQ-AQCDQDFQCKETGRCLKRHLVCNGD  118
mouseC8a    YKWTDCFPCQDKKYRYRSLLQPSKFGGTICSGDIWDEASCDSPTPCLRQ-AQCGQDFQCRETGRCLKRHLVCNGD  118
RtroutC8a   SSWSPCDSCTDKKFRFRYMEKASQFGGRQCLDSQWEELACPTAQAVCWEPDICGERFTCNATGRCVSQALRCNGE  150

                                        MACPF
GcC8a       KDCADVSDEETCEBDYPYERRTFCSDLFLIPGIEAIMTGYNILIHDVGRTVLDTG-FGGYCEYVYNGDWRELRYD  200
xenopusC8a  LDCRDSSDEEDCDAF---EHETFCKTLFPIPGAEKSVRGINILTHEDTRNVIDHNYFGGQCEYIYNGEWRELRYE  188
humanC8a    QDCLDGSDEDDCEDVR--AIDEDCSQYEPIPGSQKAALGYNILTQEDAQSVVDASYYGGQCETVYNGEWRELRYD  191
mouseC8a    NDCLDGSDESDCEDVR--VTEDDCHQYEPIPGSERAALGYNILTQEEAQSVYDAKYYGGQCETVYNGDWRKLRYD  191
RtroutC8a   VDCDDESDETDCEQVD--DRQDKCSTLLFIPGAGRGTQGFNILTGEFVDHVLDPQYYGGQCEYVYNGEWRKLIYD  223

GcC8a       SECERLYYNDDEKYYRKPYNLLTYRFEAIADSGFTIDVHNDVHELITAMKHTDSFDFAVGVQVSVVKA------A  269
xenopusC8a  PVCEQMYYSDEEKYFRKPYNFHIYQFLAPADTKMSIEIYEDSKDVVNAVKRDFSFNIGLTFGISVPEAPVGLELG  263
humanC8a    STCERLYYGDDEKYFRKPYNFLKYHFEALADTGISSEFYDNANDLLSKVKKDKSDSFGVTIGIGFAGSPLLVGVG  266
mouseC8a    PTCERLYYGEDEKYFRKPYNFLKYHFEALADTSISSEFYDDANDLFFHIKNGKSHSAGVTVGVAFVKSPVSIEVT  266
RtroutC8a   PFCENLHYNEDEKNYRKPYNFHTYRFMAQATSEGSSEYYEDMATLLKAPKTEDSFNLGVTVGIRYVEFG------  294

GcC8a       IAFGEYSTFIRNVSRFQGKDVSFVRVRTKIQTAHFKMRRYNLLLDEDMTQSLMKLP-DEYNYGMYAKFIADYGTH  343
xenopusC8a  LNYGLKTSFLKKITSFNQKNLEFVRMVTKIQTARFKMRRNLLTLDEDAMQSLMELP-DEYNYGLYAQFINDFGTH  337
humanC8a    VSHSQDTSFLNELNKYNEKKFIFTRIFTKVQTAHFKMRKDDIMLDEGMLQSLMELP-DQYNYGMYAKFINDYGTH  340
mouseC8a    GSGSKASSFLNKLNKYNEKRYGFMRVSTKIQTAQFKMRRNNIVLDEGMLQSLMELP-EQFNYGMYAKFINDYGTH  340
RtroutC8a   VSGNVESALLTNLTKYTNQELGFIPLQSKVQTAQFKMRSEGLMLHEDMYLSLMELPEEKYDFGLYSRFLNTYGTH  367

GcC8a       YYASGTMGGVYEFILVLNKKALAESDLTASEAGFCVAGSLGVVVSKGTMDVGGSV-KAKG--CRRKLEQNERSEK  415
xenopusC8a  YTTSGTMGGLVENIVVLDKEIMKKQEITASMVSHCFGASVRLSVQTEFEEIIPSL-KLSGDFCTKFERENQDNSS  411
humanC8a    YITSGSMGGIYEVVIVLDKAKMESLGITSRDITTCFGGSLGIQYEDKINVGGG----LSGDHCKKFGGGKTERAR  411
mouseC8a    YITSGTMGGIYEYVMVLDKEKMKTEGTTVDEVQKCIGGGIGIGIKDSTIEGVG----ISGEFCENSGDGDRDIRK  411
RtroutC8a   YVTQGIMGGTLEYVAVVNTTAMKTSKIDAEQLKGCLGGSIGISSPIGKTKQVEVGGKLEVKGCKGTGSYEKEMYG  442

GcC8a       SRSLVEDVLPHVLGGDLKSSAGLLGHGIPDVKMYRHWGKSLKYLPAVIDFELMPIYDLVARSKLQSVEIKQ-QNL  489
xenopusC8a  SSPAIKDVITYVIGGDSGSAGGILN--VFDGRMYRYWGRSLKYNPAVIDFEIQPIYEGLQQTGLSGIEARR-QNL  483
humanC8a    KAMAVEDIISPVRGGSSGWSGGLAQ--NPSTITYRSWGRSLKYNPVVIDFEMQPIHEVLRHTSLGFLEAKR-QNL  483
mouseC8a    KITGVEDIISRVQGGSVWGGSVLTH--NSSAITYQSWGRSLKYNPVVIDFEMQPIYQLLRHTNLGPLETRR-QNL  483
RtroutC8a   SSSLIKDIVTLVKGGSTGGSGGLLA--IKDPDTYRKWGLSLKYNPNLIEFETLPIFELVRLSTAGDHVGARPAHL  515

                    EGF                                           TSP1
GcC8a       KRAMEEYLVEFHPCRCPVCYNNGRAVLLDNVCTCECLPGYEGKACQLIKRKGP----TNGNWSCWSGWSSCQNGS  560
xenopusC8a  KRAYNEYLSEFDPCRCGPCHNNGMPMLENNVCTCLCAAGFNGPSCENTLRKDVK---ADGRWSCWSPWTQCQSGK  555
humanC8a    RRALDQYLMEFNACRCGPCFNNGVPILEGTSCRCQCPLGSLGAACEQTQTEG---AKADGSWSCWSSWSVCRAGI  555
mouseC8a    RRALDQYLMEFNACRCGPCFNNGEPILDGTNCRCQCSMGRQGLACERTVIEGLKDFKAAGHWSCWSSWSECRGGS  558
RtroutC8a   RRAWEEYLLQFNSCRCAPCRHDGIEVLSQTSCHCICKQGFRGEACEETLRKDST---TDGAWSCWGAWSSCQSGS  586

GcC8a       KQRTRSCNHPPPKDGGATCLGKNTQTKHC  589
xenopusC8a  RQRTRECNNPAPKNGGAWCLGKSLQSEPC  584
humanC8a    QERRRECDNPAPQNGGASCPGFKVQTQAC  584
mouseC8a    QERRRQCNNPPPKNGGTPCLGFNLQTQAC  587
RtroutC8a   KTPRRSCDNPQF-DGGAACLGSSSQNQPC  615
```
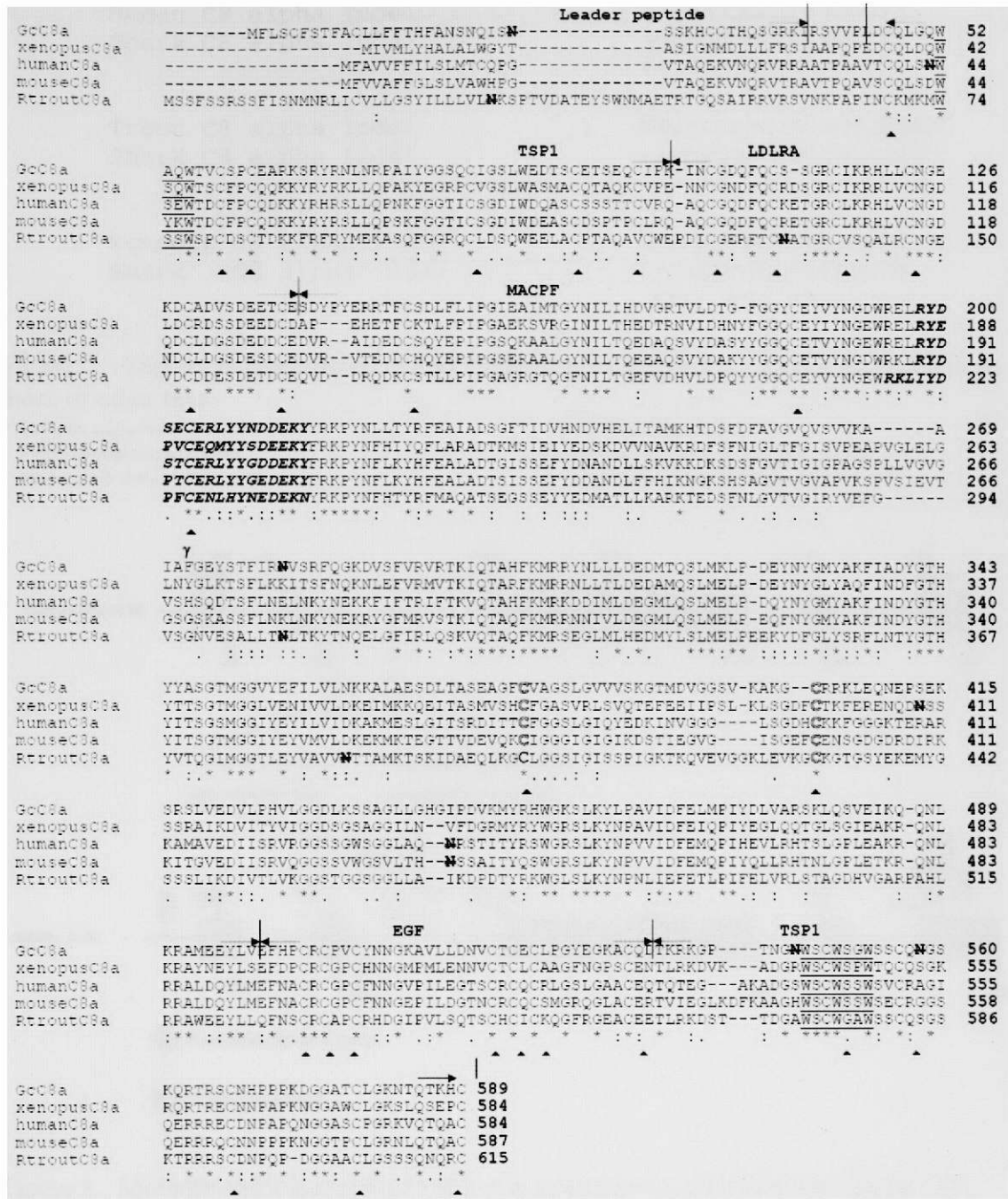
Figure 6. Full-length amino acid sequence alignment in ClustalW of GcC8α with homologues from other vertebrates: *Homo sapiens, Mus muluscus, Xenopus tropicalis, and Oncorhynchus mykiss*. The two conserved cysteine residues that are predicted to form a di-sulfide bridge and the indel sequence are highlighted in bold italics.

Legend:
* residues conserved in all six proteins
: strong group conservation
. weak group conservation
▲ cysteines conserved between mammalian C8α and GcC8α
γ C8γ disulfide bond
N putative N-linked glycosylation sites
_ putative C-mannosylated sites

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Human C8 alpha Indel          RYDSTCERLYYGDDEKY
Shark C8 alpha Indel          RYDSECERLYYNDDEKY
                              ****  ****** *****


Trout C8 alpha Indel          RKLIYDPFCENLHYNEDEKN
Shark C8 alpha Indel          ---RYDSECERLYYNDDEKY
                                 **.  **.*:**:***


Xenopus C8 alpha Indel        RYEPVCEQMYYSDEEKY
Shark   C8 alpha Indel        RYDSECERLYYNDDEKY
                              **:.  **::**.*:***
```

Figure 7: Alignment comparison of shark C8α insertion/deletion sequences (indel) with those of other taxa

* residues conserved in all six proteins   γ C8γ disulfide bond
: strong group conservation   N putative N-linked glycosylation sites
. weak group conservation   _ putative C-mannosylated sites

Figure 8. Modular and Glycosylation site map comparison of Human and Shark C8α. Schematic representation of the organization of modules/domains 5' – 3' direction in GcC8α shows it to be similar to that of human C8α in domain architecture: T1 repeats (■), LA(◆), MACPF (■), and an epidermal growth factor region (●). Potential N-linked glycosylation sites are featured as stalkcircle with an N and predicted C-mannosylation sites have a C. The GcC8α indel was aligned in ClustalW with the human C8α indel. Asterisk (*) denote residue identity and period (.) indicate similar residues.

located toward the end of the sequence in the final T1 repeat domains at amino acids 547-553 in the shark sequence in the pattern of WSCWSGW (Hofsteenge et al., 1999). A Kyte and Doolittle (1982) hydrophobicity analysis comparing GcC8α to human C8α was made in the BioEdit program (Hall, 1999); the results are shown in Figure 9. The GcC8α hydrophobicity profile suggests consistent similarities in the hydrophobic regions with the exception of regions spanning amino acids 140–190 which lies between the LA and MACPF modules, and amino acid stretches 380–395 and 462–470 within the MACPF domain. These amino acid stretches are more hydrophobic in the shark implying that GcC8α has the ability to traverse a membrane in a similar fashion as human C8α.

Gene copy analysis of GcC8α

Southern blot analysis was performed to determine the gene copy number of GcC8α in the shark genome. A single hybridizing band was detected in each of the DNA digests (enzyme digestion by *Bam*HI, *Eco*RI, *Hind*III, and *Pst*I), indicating a single gene copy of C8α in the shark genome (Figure 10).

Tissue Expression of GcC8α

The expression of GcC8α mRNA in tissues of the nurse shark was detected by RT-PCR (Figure 11). This semi-quantitative analysis revealed distinctly high levels of GcC8α transcripts in the liver, which is the primary tissue of complement protein synthesis in most organisms. The level of expression was similar to that of the β-actin control. GcC8α synthesis, albeit at lower levels, was detected in all tissues examined (kidney, brain, intestine, ovary, muscle, heart, pancreas, erythrocytes, and leukocytes),

except for the spleen, where no expression was detected. This semi-quantitative analysis

shows that nurse shark muscle tissue and erythrocytes have the highest levels of GcC8α

synthesis after liver and smaller amounts in kidney, brain, intestine, ovary, heart,

pancreas, and leukocytes. β-actin expression, included as a positive control, was
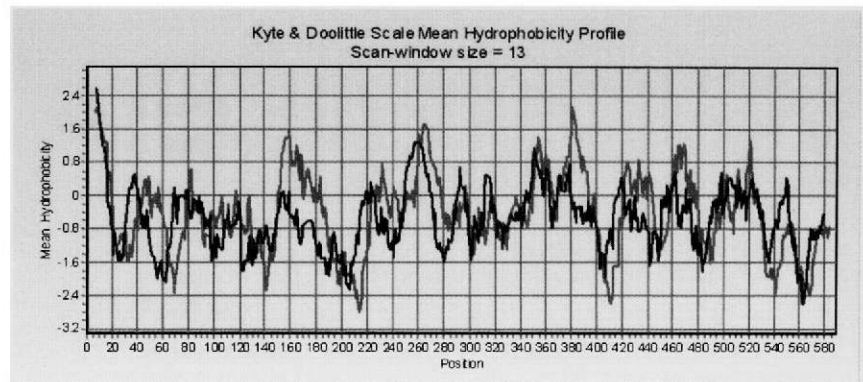
relatively uniform in all tissues examined.

Figure 9: Molecular analysis: Hydrophobicity profile of shark C8α (grey) and human
C8α (black) using the Kyte & Doolittle scale computed in the BioEdit program

Western Blot Analysis

Western blots were used to determine, using cross-reactive antibodies, the

presence in shark serum of proteins analogous to mammalian MAC proteins. Blots were

developed using goat anti-human sera to C6, C7, C8, and C9 as the primary antibody.

The secondary antibody was a rabbit anti-goat tagged with HRP.

Western blot using Anti-human C9 antisera

The Western blot using goat anti-human C9 (Figure 12) as the primary detection

antibody revealed reactive bands above 250 kDa under reducing (R) and non-reducing

(NR) conditions. This could possibly be due to cross-reactivity between the anti-human

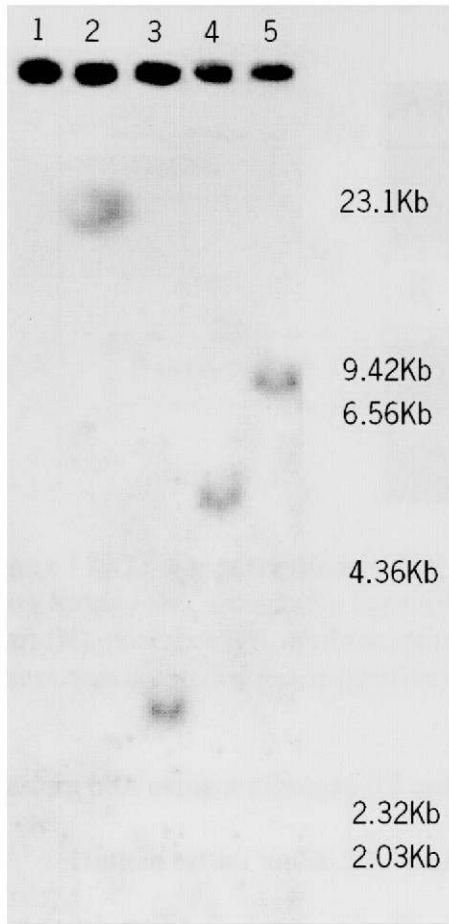C9 antibody and aggregated poly-C9 of SC5b-9. There was also a reactive band at 71

Figure 10. Southern blot analysis. Genomic DNA from shark whole blood was isolated and digested with the restriction enzymes, *Bam*H I (Lane 2), *Eco*RI (Lane 3), *Hind*III (Lane 4), and *Pst*I (Lane 5), electrophoresed, transferred to a nylon membrane and subjected to hybridization with a DIG- labeled probe. A lambda DNA/*Hind* III-digests DNA mass marker was run in lane 1, the scale is displayed to the right of the blot.

kDa in human serum under both reducing and non-reducing conditions, which

corresponds to the molecular weight of human C9. In addition, in the reduced sample of

human serum a band at ~40 kDa was detected. Reduced and non-reduced samples of

shark serum and supernatant 1 showed bands at 49 kDa and in the NR shark serum

sample a band at 68 kDa was revealed which may correspond to shark C9, suggesting

that that the shark molecule is slightly smaller than the human homologue.

Figure 11. Tissue expression of GcC8α. RT-PCR analysis of GcC8α expression in
shark kidney (K), spleen (S), brain (B), liver (L), intestine (I), ovary (O), muscle (M),
heart (H), pancreas (P), erythrocytes (E), and leukocytes (L). Expression of β-actin in
tissues was amplified as the positive control.

Western blot using antihuman C8 antiserum

Human serum under NR conditions showed a band at 151 kDa, which represents

the human C8 trimer. Two bands were also detected in the non-reduced human serum

sample at 60 and 62 kDa that most likely represent C8α and β subunits. Therefore, under

NR conditions, the full oligomer was detected along with its individual α and β

counterparts. Under reducing conditions, there were two bands at 60 and 62 for the α and

β components of human C8. Adding DTT reduced the oligomer completely to its α and

β counterparts. In the lanes with shark serum and spt1, both under-reducing and non-

reducing conditions, a faint band was present at 64 kDa, signifying either GcC8α, a C8β

-like component, or possibly both, since in mammals they are of similar size. Under the

NR conditions, a faint band is seen at ~185 kDa in all lanes with shark protein.
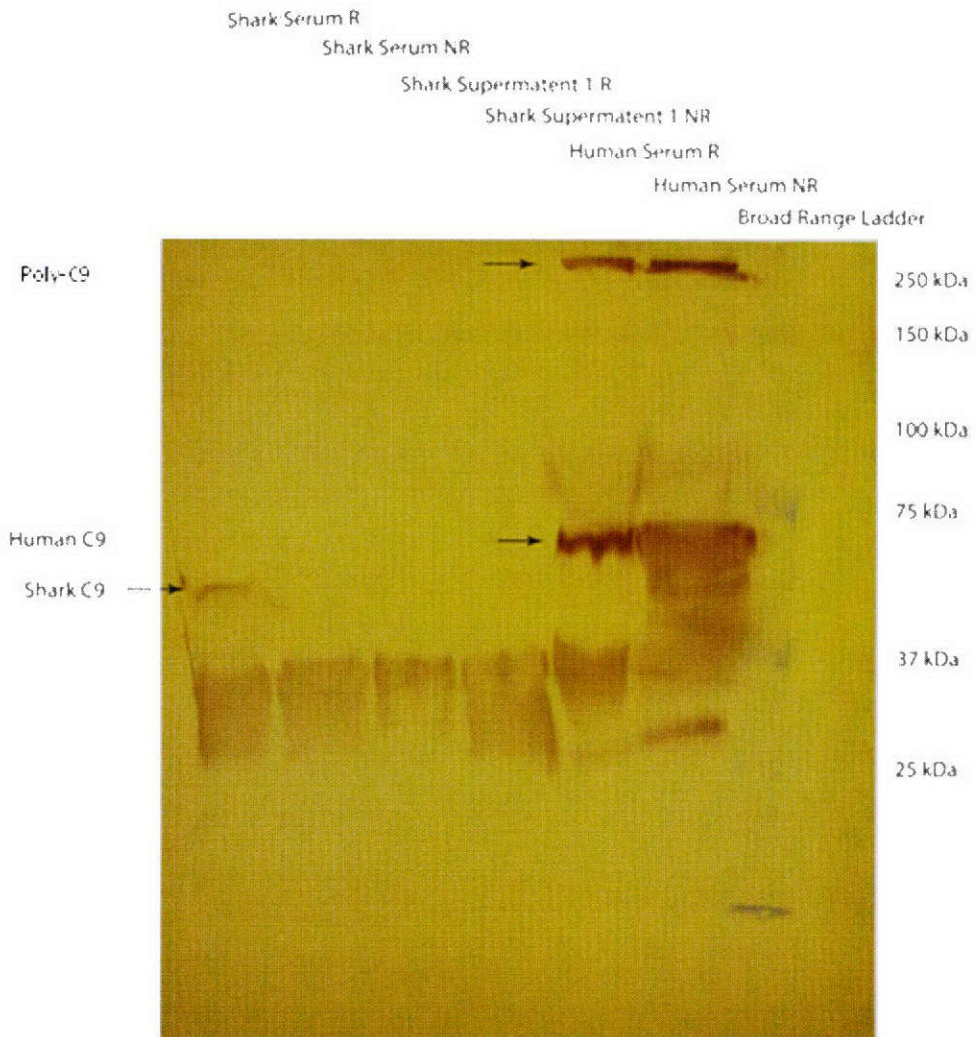

Figure 12. Western blot of C9 using whole shark serum, shark supernatent 1, and human serum samples under reducing (R) and non-reducing (NR) conditions. The blot was developed using a goat anti-human C9 primary antibody and peroxidase-tagged rabbit anti-goat Ig secondary antibody.

Western blot using antihuman C7 antiserum

The Western blot yielded a single band for human serum at 110 kDa representing human C7 under NR conditions. Since C7 is a single polypeptide a corresponding band was expected to be present in the reduced human serum sample which was not the case.
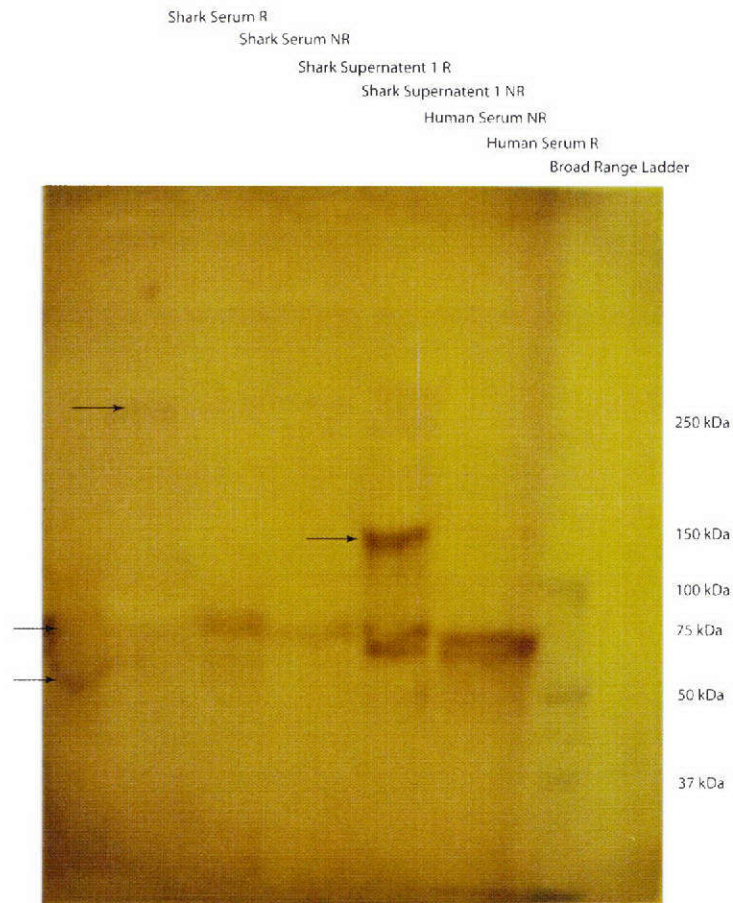

Figure 13. Western blot of C8 using whole shark serum, shark supernatent 1, and human serum samples under reducing (R) and non-reducing (NR) conditions. The blot was developed using a goat anti- human C8 primary antibody and peroxidase-tagged rabbit anti-goat Ig secondary antibody.

It is possible that DTT (the reducing reagent) may have reduced intra-protein disulfide bond(s) that changed the conformation of human C7 protein, obscuring the epitope that the anti-human C7 antibody would recognize. Furthermore, no reactive bands appeared

in any of the shark samples suggesting that either the antibody did not recognize and cross-react with a corresponding C7-like shark protein or a C7 homologue is absent in shark serum.

Western blot using anti-human C6 antiserum:

Western blot analysis of human serum, shark serum, and shark supernatant 1 using polyclonal anti-human C6 antiserum yielded no detectable bands in any of the samples run, including human serum. No reactivity with human serum was unexpected and might have been due to the dilution of the primary and secondary antisera in which the concentration of antibodies was insufficient to produce a detectable reaction.
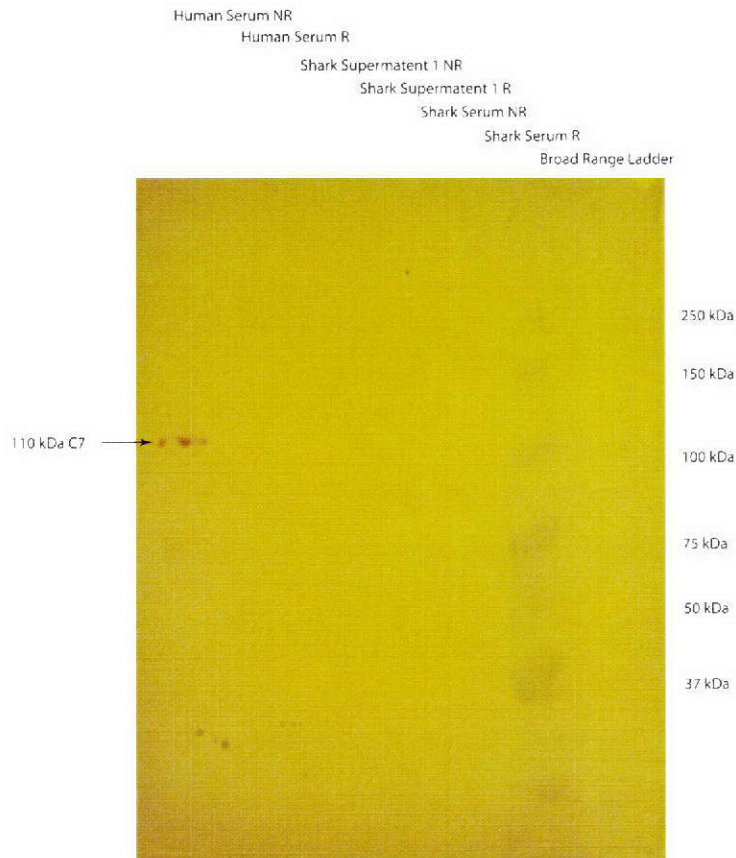

Figure 14. Western blot of C7 using whole shark serum, shark supernatent 1, and human serum samples under reducing (R) and non-reducing (NR) conditions. The blot was

developed using a goat anti-human C7 primary antibody and peroxidase-tagged rabbit anti-goat Ig secondary antibody.

CHAPTER V.

DISCUSSION

This study is based on the hypothesis that the lytic activity of shark serum is due to the presence of a functional lytic pathway that assembles a membrane attack complex similar to mammalian MAC even though in the shark, it may be executed differently due to different composition. Since there is no evidence of complement lysis and/or MAC genes or proteins corresponding to C6 and C7 in any organisms more basal than teleosts, the question remained what was the composition of shark MAC and whether it involved molecules corresponding to C6 and C7. This initial study focused on the C8α subunit of shark C8 as a first step to define shark MAC and further our understanding of the evolution of this protein complex. Furthermore, evidence was sought to determine whether C6 and C7 homologues could be detected in shark serum. The shark C8α gene was cloned, sequenced, and characterized. Western blot analysis was performed to examine shark serum for the presence of potential C6, C7, C8 and C9 homologues.

Here we report in detail the first nucleotide and deduced amino acid sequence of a full-length cDNA clone of a shark MAC gene homologus to C8α (Figure 4). The gene encoding the shark C8α homologue (GcC8α) was cloned and yielded a 2431 nucleotide sequence complete with initiation codon, stop codon, polyadenylation recognition signal, and the polyadenylation tail sequence (Figure 4).

The cloned GcC8α sequence translated into a 589 amino acid sequence (Figure 4 and 6) that exhibits 41.3% identity (Table 4) with human C8α and has the highest percent identity with the frog C8α sequence (47.4 %). Phylogenetically, GcC8α is sister taxa to

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frog C8α and that teleost C8α is more basal to both these genes, confirming that shark

C8α is more like that of the frog than bony fish (Figure 5). The shark C8α sequence has

more identity with mammalian (mouse - 42.6% and pig - 44.0%) and frog C8α sequences

than to trout (38.7%). The shark C8α sequence shares the lowest sequence identity and

similarity to chicken C8α – 36.9% and 65.4%, respectively. In terms of similarity, it was

surprising that the GcC8α sequence exhibited the highest similarity to a mammalian

sequence, mouse C8α, at 78.4%, and had the next highest percent similarity to frog and

pig C8α, 77.4% and 77.1%, respectively.

Alignments of the deduced GcC8α sequence reveal conservation of modules that

are characteristic to the MAC protein family. Structural analysis reveals that the MAC

modular architecture is conserved and organized in the same sequential order as

mammalian MAC proteins, specifically T1 (aa 47-100), LA (aa 104-139), MACPF (aa

289-498), EG (aa 523-534), and a final T1 (aa 544-588) module (Figure 8).

C8α is a unique member of MAC in that it contains an indel site that contains the

cysteine 164 (Cys[164]) residue that covalently binds C8γ (Plumb and Sodetz, 2000). In

humans, the indel region is the sequence that C8γ associates with, even when Cys[164] is

replaced by a glycine residue, meaning that there is sufficient biochemical attraction in

the indel amino acid composition to bind C8γ non-covalently (Plumb and Sodetz, 2000).

Multiple alignment (Figure 6) shows that the corresponding Cys[164] as well as the region

corresponding to human indel is conserved, which indicates that GcC8α is a C8α

ortholog and shark is the most basal organism to have this unique sequence. The shark

indel (aa 198-207) is located between the LA and the MACPF domain and contains the

conserved cysteine residue at position 203, suggesting that GcC8α may occur as a disulfide-linked α–γ dimer. This region is highly conserved between human and shark (Figure 6-8), with 88.2% identity and 94.1% similarity to the human indel sequence. This conservation of structural similarity as well as high conservation of the sequence responsible for the non-covalent binding to the C8β subunit further suggests that GcC8α might work as a trimer as C8 does in other organisms. It should be noted, however, that homologues of C8β and C8γ have yet to be described in the shark.

In humans, it has been demonstrated that all MAC proteins and perforin have two conserved cysteine residues (C7: Cys^{317} and Cys^{333}, C9: Cys^{358} and Cys^{383}, C8a: ^{346} and Cys^{370}, C8b: Cys^{324} and Cys^{349}, perforin: Cys^{236} and Cys^{258}) that form a disulfide bridge. The loop formed by this bond is suspected to be outside the membrane when human MAC is inserted into the target cell (Peitsch et al, 1990). These two pertinent cysteine residues are present in GcC8α (highlighted in black in Figure 6) and suggest a similar functional role.

All cysteine residues of human C8α form intra-molecular disulfide bonds with the exception of Cys^{164}, which forms an intermolecular disulphide bond with Cys^{40} in C8γ (Slade et al, 2006). The MAC proteins are rich in cysteine residues, and the multiple alignment (Figure 6) demonstrates that a total of 29 cysteines are conserved between elasmobranch and mammal suggesting that GcC8α potentially capable of forming, through disulfide bonds, the characteristic C8α cysteine backbone suggesting a similar folding pattern and function to mammalian, amphibian, and teleost C8α. GcC8α, however, is more cysteine-rich as it has four extra cysteine residues located toward the N-

terminal of the sequence in the leader peptide region that are not likely to be involved in GcC8α function.

Examining potential glycosylation conservation in GcC8α is interesting for two reasons: 1). Conservation of glycosylation sites between evolutionarily distant creatures can help confirm that GcC8α is indeed the shark C8α homolog and 2) gylcosylation plays an important role in biological activity, because elements like glycan location can play a role in protein folding and signal response and they structurally can interfere with activation site exposure (Wells et al., 2001). The GcC8α sequence contains two putative N-linked glycosylation sites, identified at positions different from that of mammalian C8α which has two potential N-linked glycosylation sites, at ASN^{51} and ASN^{385}. In humans, the latter is the only one suspected to actually be glycosylated (Morley and Walport, 2000). The shark sites are upstream of those in human C8α in amino acid number due to the difference in length between shark and human C8α; however, the shark glycosylation site is likely analogous to the human one in that the region it is in is conserved and is in the same module. The first potential N-linked glycosylation site in human C8α is located in the T1 module, whereas in the shark it is absent from any module and is present in the sequence upstream of that module in the 5' UTR region and, therefore, is probably not of functional significance. The second N-linked glycosylation site for both human and shark is located in the perforin-like segment, which has very high sequence conservation between mammal and shark. Two potential C-mannosylation sites were identified in the shark C8α sequence in the T1 repeats and are highly conserved in all orthologs examined (Figure 6 and 8): WAQW (aa 53–56) located in the first T1

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module and WSCWSGW (aa 547–553) in the second T1 module at the C-terminal end of the molecule. As stated above, the location and distribution of glycans in the molecule is important since glycosylation can contribute to protein folding and signal response (Wells et al., 2001).

Based on the size of the coding region and not accounting for potential glycosylation of GcC8α, the predicted molecular weight is likely to be higher than that of human C8α (589 aa versus 554 amino acid residues, respectively), which also has fewer potential N-linked glycosylation sites. Shark C8α also contains a mannosylation site that is absent in the human molecule. An earlier study (Jensen et al., 1981) estimated, from partially purified shark C8, a molecular weight closer to 185 kDa; human C8 mature protein is 152 kDa. However, the structural differences noted could account for the higher estimated molecular size of shark C8. It is also possible that the C8γ homologue in shark is a larger molecule contributing to the overall higher molecular size of shark C8.

Comparing the hydrophobicity profile of GcC8α with that of human C8α shows consistent similarities in the hydrophobic regions with the exception of regions spanning amino acids 140–190 which lies between the LA and MACPF modules, and amino acid stretches 380–395 and 462–470 within the MACPF domain. The distribution and position of hydrophobic regions through the entire coding region reveal that GcC8α has the physico-chemical properties to function in a manner similar to C8α; that is, it most likely participates in hydrophilic–amphiphilic transition and contributes to the assembly and anchoring of a MAC-like macromolecule into target membranes. These amino acid stretches are more hydrophobic in the shark implying that GcC8α has the ability to traverse a membrane. Although there are small differences in the hydrophobicity profiles

of human and shark C8α, complement of both species still achieves MAC lysis. The lesions formed by shark MAC are smaller in diameter than those formed by mammalian MAC. Mammalian MAC lesions are 100 Angstroms in diameter. Membrane holes produced by shark MAC are 80 Angstroms in diameter, i.e., a difference of 20 Angstroms. However, ultra-structural studies of shark MAC lesions show them to be indistinguishable from those made by guinea pig complement (Jensen et al., 1981).

To assess gene copy of GcC8α, Southern blot analysis was performed. GcC8α exists as a single gene in the shark. Since C8α and C8β of mammals and other vertebrate species (teleosts) are very similar in sequence homology, extra care was taken to design a Southern blot probe to correspond to a region of C8α that did not overlap with any sequence in C8β (should such a homologue be present in the shark) to ensure that the Southern blot data reflects gene copy of GcC8α only. In several teleost species, some complement genes are present in several isoforms (Kato et al., 2003; Kuroda et al, 1996; Nakao et al., 2002; 1998; Sunyer et al., 1996, 1997,1998; Chondrou et al., 2008; Gongora et al., 1998; Papanastasiou and Zakardis, 2005; Papanastasiou et al, 2007). This may be due to a suspected third round of genome duplication occurring during the emergence of bony fish (Ohno, 1970). Similarly, in elasmobranchs certain complement genes are present in multiple forms, such as GcC3-1 and GcC3-2 (Smith, unpublished); GcBf/C2-1 and 2 (Shin et al., 2007); and GcIf-1, -2, -3 and -4 (Shin et al., 2009) in the nurse shark and TrscBf-A and -B in the banded houndshark (*Triakis scyllia*) (Terado et al., 2001; 2002). Thus far there has been no evidence of MAC genes having multiple isoforms in any organism so far studied, and the phenomena of multiple gene copy of complement

genes seems to be sequestered to the genes encoding proteins of the three activation pathways (CP, AP, LP).

To examine the expression profile of GcC8α, RT-PCR revealed that GcC8α is synthesized by several tissues in the shark, with the highest expression in the liver. Other tissues that synthesized GcC8α were kidney, brain, intestine, ovary, muscle, heart, pancreas, erythrocytes, and leukocytes. Interestingly, the expression in peripheral blood cells is higher in erythrocytes than in leukocytes, which indicates that nucleated erythrocytes of shark are transcriptionally active. Multiple organ/cell expression of C8α is not seen in mammals, where C8α is synthesized primarily in the liver; however, in other vertebrate species such as trout, C8α and C8β are expressed in several tissues (Kazantzi et al., 2003; Papanastasiou and Zakardis, 2006). Taken together these observations suggest that poikilothermic vertebrates synthesize complement proteins more ubiquitously than mammals. As the complement system evolved, the tissue sites for complement synthesis may have been reduced through evolution; however, the liver (hepatopancreas in some species) remains likely the main site of complement protein synthesis in vertebrates.

Phylogenetic analysis of GcC8α provides insight into the evolution of the MAC family of proteins. Two main theories attempt to explain the evolution of this significant gene family. Phylogenetic analyses by Mondragon-Palomino et al. (1999) using MAC amino acid sequences supports the view that C6 and C7 are earlier in origin and were followed by the emergence of C8 then C9. Mondragon-Palomino et al. (1999) do not present data as to the whether C8α or β is of earlier derivation, and suggest that the

terminal complex proteins (C6 through C9) originated from a single ancestral gene composed of complex modular structure. They suggest that a series of gene duplications and loss of structural modules resulted in complement proteins that make up the MAC protein family. In contrast, Podack et al. (1991) hypothesize that, due to the similarity of function, size, and sequence, C9 emerged first from a gene duplication event of an ancestral gene common to both perforin and the MAC family proteins. Podack et al. (1991) further speculate that following C9 emergence, C8, C7, and C6 successively emerged through later gene duplication events and developed increasing modular complexity and size. Kauffman et al.'s (1993) hypothesis is somewhat different, although it also supports the C8/C9 faction as originators of MAC. After distance analyses of human MAC components and perforin C8α Kauffman et al. (1993) show that C8α and β have a closer phylogenetic distance to perforin than to C6, C7, and C9, maintaining that MAC arose from a fundamental C8-like building block. The phylogenetic analysis performed in this study supports the hypothesis that C8 and C9 are derived from a common ancestor and represent an early duplication event that most likely predated C6 and C7. Although C6-like molecules have been described for amphioxus (Suzuki et al., 2000) and *Ciona* (Wakoh et al., 2004), their role as complement proteins remains unconfirmed. Molecular analysis of the C6-like gene described for amphioxus reveals a 5' C6 modular structure with a 3' end that is missing key modules characteristic of C6. This could also be interpreted to be an early C8-like molecule before loss of the extra T1 module at the 5' end. Furthermore, in *Ciona* the C6-like gene is expressed as a cell surface receptor and whether it has a complement function (Wakoh et al., 2004; Azumi et al., 2003) in either organism is unknown. This amphioxus C6 gene has not been shown to

be expressed or functional and may be C6-like only in sequence. Amphioxus C6 is also comparable in size to C8 (α and β chains) and C9.

The phylogeny constructed during this investigation showed that GcC8α forms a clade with C8α sequences from other taxa and is sister taxa with frog C8α. The tree also shows that the C8 complex has diverged from a common ancestor with C9. Human, mouse, cow, cat and woodchuck perforin sequences were used as an out group. Confidence in the branch points was validated by 1000 bootstrap replications. The tree also shows that the C8 complex has diverged from a common ancestor with C9. According to our phylogenetic analysis C8α and C8β arose from C9 followed by C7, then C6. Since the presence or absence of C7 and/or C6 homologues in shark remains to be resolved it is premature to conclude that shark MAC only consists of C5b, C8, and C9. C6 and C7 are essential to teleost, amphibian, and mammalian taxa or MAC function. If it were to be determined that one or the other of these two proteins was in fact absent in shark, the data would support Podack et al.'s (1991) hypothesis since C5 (Graham et al., 2009), C8, and C9 homologues are, so far the only MAC proteins confirmed for the shark.

The Western blot using goat anti-human C9 as the primary detection antibody revealed reactive bands above 250 kDa under reducing and non-reducing conditions. This is likely due to cross-reactivity between the anti-human C9 antibody and aggregates of proteins possibly poly-C9 or SC5b-9. There was also a reactive band at 71 kDa under both reducing and non-reducing conditions, which corresponds to the molecular weight of human C9. Anti-human C9 antibodies detected a band at ~40kDa in reduced sample of human serum. Shark serum and supernatant 1 under reducing and NR show bands at

49 kDa and the shark serum under NR conditions revealed a band at 68 kDa. This band may represent shark C9 and suggests that the shark C9 may be slightly smaller than the 71 kDa human homologue.

A band at 151 kDa in human serum was revealed in Western blots using anti-human C8 antibodies which represents the human trimeric C8 molecular complex. Two additional bands were also detected at 60 and 62 kDa representing C8 α and β chains. These bands were also present in reduced human serum, however the full oligomer was detected only in the non-reduced serum sample, suggesting that DTT reduced the oligomer completely to its α and β counterparts. In the lanes with shark serum and shark sptI, both under-reducing and non-reducing conditions, a faint band was present at 64 kDa, signifying either GcC8α, a C8β-like component, or both. Under the NR conditions, a faint band could be seen at ~185 kDa in all lanes with shark protein. The anti-human C8 antibody did not detect C8γ in the human or shark fluids, suggesting that the C8 antibody was specific for epitopes that are similar on C8α and C8β but not C8γ. Whereas C8α and β are so similar a single antibody would likely detect both and does in this instance. It is not surprising that C8γ was undetected as it is a lipocalin and small such that if the shark has C8, it would not likely be detected by this test.

Only one band is visualized on the C7 Western blot in the lane with the NR human serum at 110 kDa representing human C7. This was absent when human serum was reduced. Although C7 does not exist as a covalently bound multimer, it is cysteine rich and has many internal disulfide bonds which were probably reduced by the DTT. This would change the topography of the protein and the integrity of its epitope and

would account for non-reactivity with anti-C7 antiserum made to native protein. The same antibody failed to detect protein in any of the shark samples. This could mean that C7 in shark (should it be present) is antigenically distinct from its mammalian counterpart, or that the molecule is absent in sharks.

In Western blot using antiserum to human C6 no protein bands were detected in any of the samples run including human serum, suggesting that the antibody concentration was too low to achieve a detectable reaction of the antibody with its target epitope. Another possibility is that the antibody was specific for an epitope of the folded C6 and that the human serum was sub-optimal in that the disulfide bonds had been reduced over time or through handling obscuring the epitope the antibody was specific for. The question of C6 homologue in the shark remains unresolved.

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