Complement component C8γ is expressed in human fetal and adult kidney independent of C8α

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Abstract Human complement component C8γ is an unusual complement factor since it shows no homology to other complement proteins but is a member of the lipocalin superfamily. So far, it has been found exclusively in plasma, covalently linked to C8α by disulfide bridging. We have used dot blot and Northern blot analyses of a large number of different human tissues to survey systematically the expression pattern of C8γ. Our experiments clearly showed that besides in liver, this gene is also expressed in fetal and adult kidney. Renal expression of C8γ is not dependent on C8α expression, since we could not detect C8α expression in kidney. Thus its physiological function is not restricted to a specific action in association with complement components. As a prerequisite for further characterization of the structure and binding activities of the uncomplexed C8γ, we have expressed the encoding cDNA in *Escherichia coli*. To increase the probability for proper folding of the characteristic intramolecular disulfide bridge the recombinant protein was produced by secretion to the periplasm.

Key words: Complement component C8γ; Lipocalin; Kidney; Expression; Recombinant protein

1. Introduction

Complement component C8, the penultimate member of complement membrane attack complex (MAC), is composed of three different polypeptides, α (M_r = 64 000 Da), β (M_r = 64 000 Da) and γ (M_r = 22 000 Da) encoded by different genes [1]. In plasma the C8 complex is arranged as a disulfide-linked C8α-γ dimer which is non-covalently associated with C8β [2]. C8γ is structurally unrelated to any other complement component but was found to be a member of the lipocalin superfamily [3,4]. Members of this expanding family are small mainly secretory proteins which are characterized by their ability to bind or transport hydrophobic molecules and by their common structure [5]. Within the highly divergent lipocalin family C8γ shows significant amino acid sequence homology with α_1-microglobulin and the closely related protein HC (human complex-forming glycoprotein).

Whereas defined roles in the formation and function of MAC have been proposed for C8α and C8β [6], the function of C8γ remains unclear. It is neither required for the synthesis or function of C8, nor for the formation and lytic activity of MAC [7]. Since retinol binding was reported for a number of other lipocalins [8–10], it was suggested that C8γ might function as a retinol carrier. Previous studies indeed indicated a weak binding of retinol and retinoic acid to C8α-γ [11]. However, this binding was not found in a more recent and reliable study [7], thus a retinol carrier function remains doubtful. Another postulated function of C8γ, namely to shield or otherwise protect hydrophobic regions of C8α from premature membrane interaction during biosynthesis processing, could not be verified [7].

A major prerequisite for further investigations concerning the biological function of C8γ is to know whether it is found exclusively associated with complement components, or is expressed independently in other organs or tissues. Therefore we have started investigations concerning the expression of C8γ using PCR and Northern analysis. Since our studies have clearly shown expression of C8γ in kidney independent of C8α synthesis, we have expressed the C8γ encoding cDNA from kidney in *Escherichia coli*. This uncomplexed form of C8γ will be used in further studies concerning ligand binding and structural analysis.

2. Materials and methods

2.1. RNA preparation

Total RNA from human liver and kidney was extracted by RNazol B (Cinna/Biotecx) which is based on the method developed by Chomczynski and Sacchi [12]. Poly(A)$^+$ RNA was prepared using oligo-dT cellulose. RNA was resuspended in diethylpyrocarbonate-treated water and was examined on 1.2% agarose-formaldehyde gels.

2.2. Reverse transcription, RT-PCR analysis and molecular cloning

5 μg of total RNA was reverse transcribed using 0.5 μg of $(dT)_{12-18}$ as primer together with 200 units of Superscript II reverse transcriptase, according to the manufacturer's specifications (Gibco BRL).

An aliquot (10%) of the cDNAs was amplified by PCR using the primers E2C8γU: 5′-TTTGCAGGGACCTGGCTCCT-3′ and E2C8γL: 5′-AGCTTTCGGAAGGTACTGAC-3′ corresponding to nucleotides 200–219 and 317–336 of the C8γ cDNA [1] or primers C8a 7-1: 5′-CACAAGAATCTTCACAAAGG-3′ and C8a 7-2: 5′-GTCAATCACCAGGATATATTC-3′ for C8α [13]. PCR amplification was performed by the following reaction conditions (final concentrations): 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.01% gelatine, 0.1% Triton X-100, 3 mM $MgCl_2$, 0.2 mM dNTPs, 30 pmol of primers in a total volume of 50 μl. Each cycle entailed denaturation at 95°C for 60 s, annealing at 55°C for 60 s and primer extension at 72°C for 60 s. PCR products were analyzed on 2% agarose gels stained with ethidium bromide. As a control the experiment was performed using RNA without reverse transcription.

The resulting PCR fragment was purified by electrophoresis in a 2% agarose gel and ligated into the pGEM-T vector (Promega Corp., USA) using the manufacturer's protocol. The nucleotide sequence of both strands of the double-stranded DNA was determined by cycle sequencing using Taq polymerase [14].

2.3. Northern blot analysis

Total RNA isolated from human liver and kidney and commercially available multiple tissue RNA blots (Clontech) were used for

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Abbreviations: C, complement; IPTG, isopropyl-β-D-thiogalactopyranoside; MAC, membrane attack complex; OmpA, *Escherichia coli* outer membrane protein A; NTA, nitrilotriacetic acid

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Northern blot analysis. Blots were hybridized for 18 h at 42°C in 'high SDS' solution: 7% SDS, 50% formamide, 5×SSPE, 10% blocking reagent (Boehringer, Germany), 0.1% lauroylsarcosine and 50 μg/ml salmon sperm DNA, with a ^{32}P-labeled C8γ DNA probe. Blots were washed for 2×15 min in 1×SSC, 0.1% SDS at room temperature, and for 2×15 min in 1×SSC, 0.1% SDS at 55°C.

2.4. Prokaryotic expression of the C8γ cDNA

For bacterial expression the region of the kidney-specific C8γ cDNA which encodes the mature protein was selectively amplified by PCR using the primers C8gpQE-5: 5′-AAGCCTCAGAGGC-CACGCCGGCCC-3′ and PQEC8NG: 5′-CGCGGATCCCTCCT-CACTTCGTCCAGG-3′. The latter primer incorporates sequences for a unique *Bam*HI site (Fig. 4). To achieve a 5′-blunt ended PCR fragment we used PfuTurbo DNA Polymerase (Stratagene, USA). The PCR product was digested with *Bam*HI, purified by agarose gel electrophoresis and ligated to a chemically synthesized DNA fragment encoding the signal peptide sequence of *E. coli* OmpA. The OmpA fragment contained a 5′-*Sph*I cleavage site and a 3′-blunt end. After ligation the OmpA-C8γ fusion was cloned into the *Sph*I-*Bam*HI site of pQE-70 (Qiagen Inc., USA) and *E. coli* M15 [15] was used as a host. At an OD_{600} of about 0.5, IPTG was added to a final concentration of 2 mM and the cells were grown for another 5 h. For purification of the recombinant protein from periplasm, cells were centrifuged and the pellet was resuspended in 1 M NaCl, 1 mM EDTA, 40 mM sodium phosphate (pH 7.5) and kept on ice for 30 min [16]. The spheroplasts formed during this step were separated by centrifugation and the supernatant was dialyzed overnight against 1 mM NaCl, 40 mM sodium phosphate (pH 7.5) in order to remove the EDTA. The resulting protein solution was directly applied to affinity chromatography on Ni-NTA resin (Qiagen Inc., USA) exactly as described by the supplier and the recombinant protein was eluted by a gradient of imidazole in column buffer. The eluted protein was characterized by SDS-PAGE and N-terminal amino acid sequencing by a modified Edman degradation protocol on a gas/liquid phase sequenator [17].

3. Results

3.1. Expression of C8γ in human kidney

To survey systematically the tissue-specific expression of C8γ, a Northern dot blot analysis was performed using a commercially available RNA filter on which poly(A)$^+$ RNAs from 50 different human tissues (reported in the legend of Fig. 1) were immobilized. The amount of dotted RNAs was normalized to the expression level of eight housekeeping genes by the manufacturer, thus allowing determination of the relative expression level of a target mRNA in different tissues. As shown in Fig. 1 strong hybridization signals were obtained with liver and kidney RNA from both adult and fetal tissues (Fig. 1, E 1,2 and G 3,4) using a radiolabeled 137 bp cDNA fragment of C8γ. No other hybridization signal was detected that was significantly above the background. Estimation of the level of expression, as determined by phosphor imaging, revealed that expression of C8γ in kidney was equivalent to that in liver.

To test whether the renal C8γ is coexpressed with C8α or is expressed independently, we performed PCR analysis using primers specific for C8α or C8γ. From Fig. 2 it is evident that a C8γ PCR product, but no C8α product was obtained from kidney RNA, whereas from liver RNA a product was obtained for both of the genes. We also hybridized a Northern blot containing RNA from several fetal tissues (brain, lung, liver and kidney). With the C8γ probe a hybridization signal was detected with liver and kidney RNA (Fig. 3A), whereas with the C8α gene probe a signal was only seen with liver RNA (Fig. 3B). Similar to the tissues from adult, quantification of the signal intensity on these blots indicated that

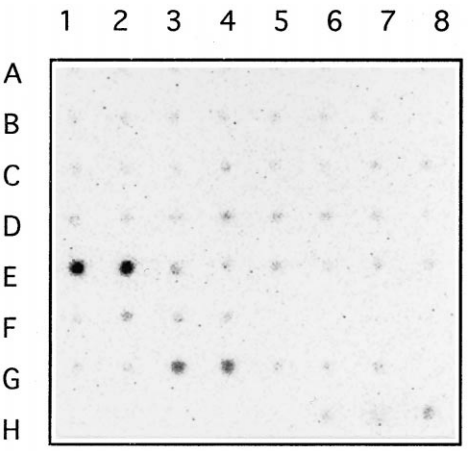

Fig. 1. Human multiple tissue dot blot (Clontech) containing poly-(A)$^+$ RNAs from 50 different tissues immobilized in separate dots was hybridized with a cDNA probe for human C8γ. Row A: 1, whole brain; 2, amygdala; 3, caudate nucleus; 4, cerebellum; 5, cerebral cortex; 6, frontal lobe; 7, hippocampus; 8, medulla oblongata. Row B: 1, occipital lobe; 2, putamen; 3, substantia nigra; 4, temporal lobe; 5, thalamus; 6, subthalamic nucleus; 7, spinal cord. Row C: 1, heart; 2, aorta; 3, skeletal muscle; 4, colon; 5, bladder; 6, uterus; 7, prostate; 8, stomach. Row D: 1, testis; 2, ovary; 3, pancreas; 4, pituitary gland; 5, adrenal gland; 6, thyroid gland; 7, salivary gland; 8, mammary gland. Row E: 1, kidney; 2, liver; 3, small intestine; 4, spleen; 5, thymus; 6, peripheral leukocyte; 7, lymph node; 8, bone marrow. Row F: 1, appendix; 2, lung; 3, trachea; 4, placenta. Row G: 1, fetal brain; 2, fetal heart; 3, fetal kidney; 4, fetal liver; 5, fetal spleen; 6, fetal thymus; 7, fetal lung. Row H: 1, yeast total RNA 100 ng; 2, yeast tRNA 100 ng; 3, *E. coli* rRNA 100 ng; 4, *E. coli* DNA 100 ng; 5, poly(A)$^+$ 100 ng; 6, human c_0t1 DNA 100 ng; 7, human DNA 100 ng; 8, human DNA 500 ng.

C8γ expression in fetal kidney is equivalent to that in fetal liver.

Since it is known that within the lipocalins several members of one specific protein family could be encoded by different, but very closely related genes [18,19], we cloned the entire kidney-specific C8γ transcript to perform sequence analysis. Therefore, poly(A)$^+$ RNA from human kidney was reverse-transcribed and used as a template for PCR with oligonucleotide primers derived from the 5′- and 3′-non-translated regions of the C8γ cDNA. The resulting 709 bp DNA fragment was ligated into a pGEM-T cloning vector and two positive clones from independent PCR reactions were subjected to DNA sequencing. The nucleotide sequence (data not shown) of the cloned fragments was 100% identical to the liver-specific C8γ cDNA sequence [1], thus indicating that C8γ from liver and kidney are products of a single gene.

3.2. Prokaryotic overexpression of C8γ

Recently, human liver-specific C8γ was expressed in insect cells and COS-7 cells mainly to investigate the association with other C8 factors and to study the function of C8γ in context with complement action [7]. However, since our major goal for producing a recombinant protein is to perform structural studies using NMR technology, which needs production of large amounts of labeled protein, we decided to use *E. coli* as an expression system. Although successful cytoplasmic production of lipocalins in *E. coli* was reported [20,21], two aspects had to be considered: on the one hand, C8γ, as a component of MAC, might be toxic for *E. coli*; on the other hand,

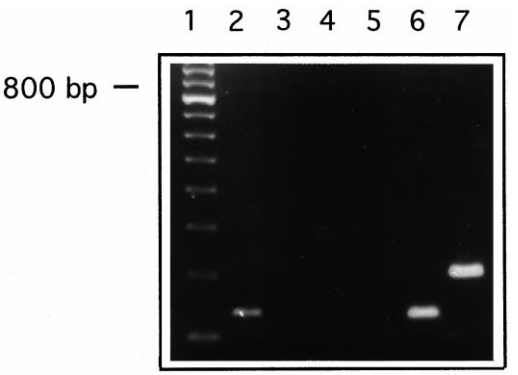

Fig. 2. Agarose gel electrophoresis of PCR products. Lane 1: 100 bp DNA ladder; lane 2: kidney cDNA as template and C8γ primers; lane 3: kidney cDNA template and C8α primers; lanes 4 and 5: same as lanes 2 and 3 but without reverse transcription; lane 6: liver cDNA as template and C8γ primers; lane 7: liver cDNA as template and C8α primers. 8 μl of the PCR reaction was applied to each lane.

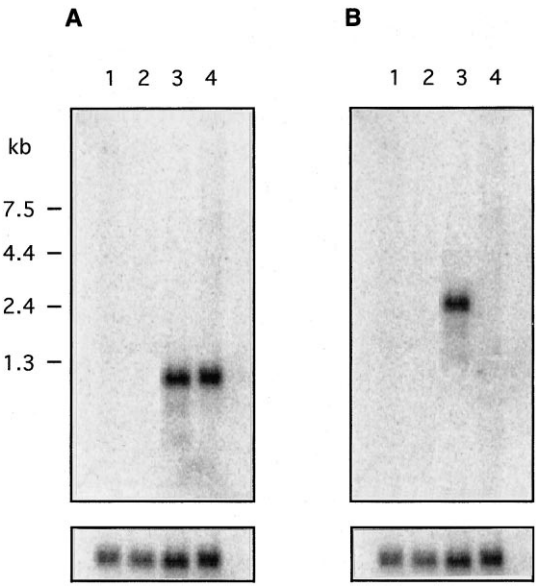

Fig. 3. Northern analysis of a human fetal multiple tissue blot. Lane 1: fetal brain; lane 2: fetal lung; lane 3: fetal liver; lane 4: fetal kidney; each lane contained approximately 2 μg of poly(A)$^+$ RNA. A: Hybridized to a human C8γ cDNA probe. B: Hybridized to a human C8α cDNA probe. For loading control the blot was stripped and hybridized to a human GAPDH cDNA probe (lower).

C8γ has an intramolecular disulfide bond and a free Cys and might therefore be incorrectly folded when expressed in the cytoplasm. Therefore, we fused the *E. coli* OmpA signal peptide encoding DNA to the DNA sequence encoding the mature C8γ (Fig. 4) in order to achieve periplasmic secretion. Since the N-terminal amino acid residue of the native mature C8γ could not be identified [1], we used an algorithm [22] indicating Gln^{21} and Lys^{22} as most probable N-terminal amino acids for the mature protein. For the recombinant C8γ we chose Lys^{22} as the N-terminal amino acid because when fused to the OmpA signal sequence this amino acid gives the most preferential cleavage site in *E. coli* as calculated [22]. For facile purification the recombinant protein was produced with a 6×His tag attached to the C-terminus. The OmpA-C8γ fusion construct was cloned into a modified pQE vector and transfected into *E. coli* strain M15. SDS-PAGE analysis of cell extracts after induction of heterologous gene expression by IPTG indicated the presence of an additional protein band with an apparent M_r of 21 kDa (Fig. 5), which is in accordance with that calculated from the deduced amino acid sequence. After purification by affinity chromatography using a Ni-NTA resin [23] and gel filtration chromatography, the protein was subjected to N-terminal amino acid sequence analysis and the sequence found was KPQRPRRPAS, which is identical to the deduced amino acid sequence. In addition, this result demonstrated correct cleavage of the signal sequence. A preliminary characterization by SDS-PAGE, native

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OmpA signal peptide coding sequence on vector:

    Sph I
   GCATGCGAAAGACAGCTATCGCGATTGCAGTGGCACTGGCCGGCTTCGCTACCGTAGCGCAGGCC
   ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
   CGTACGCTTTCTGGTCGTAGCGCTAACGTCACCGTGACCGGAAGCGATGGCATCGCGTCCGGTCG
     ---TT-----------------------------------------------------------
-21 MetArgLysThrAlaIleAlaIleAlaValAlaLeuAlaGlyPheAlaThrValAlaGlnAla -1
       Lys


5`- and 3´-end of the coding sequence of C8-gamma:

                                                              BamH I
   AAGCCTCAGAGGCCACGCCGGCCCGCA.............CCTGGACGAAGTGAGGAGGGATCC
   :::::::::::::::::::::::::::               :::::::::::::::::::::::
   TTCGGAGTCTCCGGTGCGGCCGGGCGT.............GGACCTGCTTCACTCCTCCCTAGG
 +1 LysProGlnArgProArgArgProAla.............LeuAspGluValArgArg      +182
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Fig. 4. Construction of the OmpA-C8γ fusion for expression in *E. coli* and secretion into the periplasm. The *E. coli* OmpA signal peptide encoding DNA was chemically synthesized containing a *Sph*I restriction site on the 5′-end and a blunt end at its 3′-end. In order to create the *Sph*I site and use the original start codon, the second codon AAA was mutated to CGA, resulting in an exchange of Lys for Arg. After *Sph*I cleavage this fragment was ligated to the 5′-blunt ended C8γ DNA fragment encoding the mature protein. Since the C8γ fragment contained a 3′-*Bam*HI cleavage site the fused product was inserted into a *Sph*I-*Bam*HI-digested pQE-70 vector (Qiagen Inc., USA).

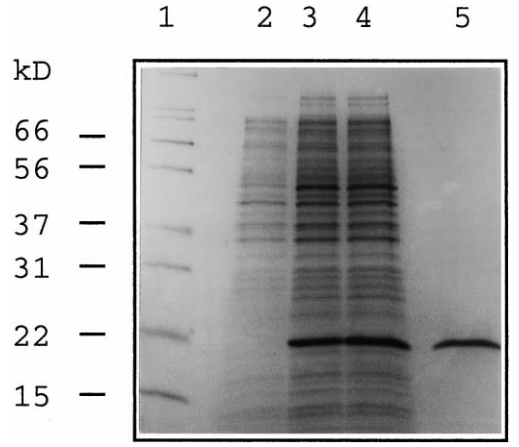

Fig. 5. Production of recombinant C8γ in *E. coli* and purification of the recombinant protein as analyzed by 0.1% SDS-14% PAGE. Lane 1: Molecular weight markers. Lane 2: *E. coli* cell extract prior to induction. Lanes 3 and 4: *E. coli* cell extracts after induction with IPTG for 3 and 5 h. Lane 5: 5 μg of recombinant protein from periplasm after purification by Ni-NTA affinity chromatography. The gel was stained with Coomassie blue.

gel electrophoresis and circular dichroism spectroscopy indicated that the recombinant protein is homogeneous and seems to be properly folded (data not shown).

4. Discussion

The data presented in this work demonstrate for the first time that C8γ, which was so far characterized as an intrinsic component of the terminal complement factor C8 in plasma, is expressed in human kidney. Although some members of the components of the classical and alternative activation pathways have been found to be synthesized in kidney [24,25], to our knowledge this is, in general, the first clear evidence for renal synthesis of a terminal complement component. The C8γ-specific mRNA synthesis in kidney is suggested to be constitutive rather than induced, because we used RNA from very different sources, including fetal RNA, and found expression in all samples. Our experiments clearly demonstrate that C8γ expression in kidney is independent of C8α, since we could not detect C8α expression in human kidney even when using the high sensitivity of PCR. Therefore, it is clear that the biological function of C8γ is not restricted to a specific role in complement-mediated cell lysis in association with C8 members, but is suggested to be more general. In this context, it is interesting to note that C8γ does not seem to be required for synthesis or function of C8α, nor is it essential for the foundation and lytic activity of MAC; however, it significantly enhances C8 hemolytic activity [7].

Based on sequence analysis, genomic structure and secondary structure, C8γ is a member of the lipocalin protein family [4,8]. Although binding of small lipophilic compounds, such as retinol or retinoids, is a typical feature of several lipocalin members [9–11], a specific retinol binding activity could not be observed with C8γ [7]. Nevertheless, additional binding studies using other lipophilic ligands have to be performed. These studies should include delipidation experiments, since endogenous ligands bound to the protein might disturb the binding assays.

Our observation that C8γ is expressed in kidney could provide novel hints to the function of this protein. Consistent with its expression in kidney and complex formation with C8α, C8γ might exhibit protective action through scavenging of potentially harmful lipophilic compounds, as suggested for other lipocalin members [26]. Such harmful products could accumulate in kidney as a direct result of the host defence in response to microbial infection or the cellular release during inflammation. A protective function of C8γ might also be of relevance in connection with C8 thereby reducing the inflammatory response when the MAC is deposited on host cell membranes, thus allowing tissue recovery from immune attack [3].

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