Isolation, Primary Structure, and Evolution of the Third Component of Chicken Complement and Evidence for a New Member of the α_2-Macroglobulin Family[1]

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Although the third component of complement, C3, has been isolated and its primary structure determined from most living classes of vertebrate, limited information is available on its structure and function for aves, which represent a significant stage in complement evolution. In this study, we present the complete cDNA sequence of chicken C3, the cDNA sequences of the thioester region for two chicken α_2-macroglobulin (α_2M)-related proteins, a simplified method for purifying chicken C3, and an analysis of the C3 convertase and factor I-mediated cleavages in chicken C3. Using the reverse-transcriptase PCR, with degenerate oligonucleotide primers derived from two conserved C3 sequences (GCGEQ$^N/_T$M, TWLTA$^Y/_F$V) and liver mRNA as template, we isolated three distinct 220-bp PCR products, one with a high degree of sequence similarity to C3 and two to α_2M and pregnancy zone protein from other species. The complete cDNA sequence of chicken C3 was obtained by screening a chicken liver λgt10 library with the C3 PCR product and probes from the 5′ end of the partial-length C3 clones. The obtained sequence is in complete agreement with the protein sequence of several tryptic peptides of purified chicken C3. Chicken pro-C3 consists of an 18-residue putative signal peptide, a 640-residue β-chain (70 kDa), a 989-residue α-chain (111 kDa), and an RKRR linker region. It contains an internal thioester and three potential *N*-glycosylation sites, all in the α-chain. The convertase cleavage site, predicted to be Arg-Ser, was confirmed by sequencing the zymosan-bound C3 fragments generated upon complement activation. NH_2-terminal sequencing of the purified C3 chains showed that 1) pro-C3 is indeed cleaved at the RKRR linker sequence to generate the mature two-chain molecule, and 2) the β-chain of chicken C3 is blocked. The deduced amino acid sequence shows 54, 54, 54, 53, 52, 57, and 55% amino acid identities to human, mouse, rat, guinea pig, rabbit, cobra, and *Xenopus* C3, respectively, and an identity of 44, 31, and 33% to trout, hagfish, and lamprey C3, respectively. The identities to human C4, C5, and α_2M are 31, 29 and 23%, respectively. A phylogenetic tree for C3, C4, C5, and α_2M-related proteins was constructed based on the sequence data and is discussed. *The Journal of Immunology*, 1995, 154: 2164–2174.

C3, the third component of complement, is the most abundant complement protein in vertebrate blood. It has been purified from the plasma of several representative classes of vertebrates, with the human molecule being the best characterized (1, 2). C3 plays a critical role in both pathways of complement activation by interacting with numerous other complement proteins. In addition, its interactions with a variety of cell surface receptors make it a key participant in phagocytic and immunoregulatory processes, and its interactions with proteins from foreign pathogens may provide a mechanism by which these microorganisms evade complement neutralization (1, 2). In all of the species that have been analyzed, with the exception of lampreys, C3 is composed of two chains linked by a disulfide bond and noncovalent forces (3), contains a thioester in the α-chain, and is glycosylated on the α-, β-, or both chains (4). The complete primary structures of human (5), guinea pig (6), mouse (7, 8), rat (9), hagfish (10), lamprey (11), cobra (12), and trout C3 (13), and partial primary sequence of rabbit (14) and *Xenopus* (15) C3 have been determined. Significant information linking the structural elements of C3 to its functions has been obtained by identifying and characterizing the conserved sites in various species (1, 3).

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The aves represent a significant stage in complement evolution. Although the complement system of birds (including chickens) is composed of both alternative and classical pathways, previously unsuccessful attempts to isolate C2 (16) have led to the speculation that in birds the protein factor B of the alternative pathway functions in both pathways. If this is indeed the case, then chicken complement represents a stage in complement evolution before the gene duplication that gives rise to factor B and C2; the complement proteins that form the C3 convertase would be shared by both pathways. Koppenheffer (17) has recently found that the terminal components in chicken serum can be activated directly by C1 with interaction of an intermediate component through a Ca^{2+}-dependent mechanism. The physiologic role of this pathway is not known; it may represent a vestigial activation pathway.

The chicken proteins C1q (18), factor B (16), and C3 (19) have been isolated and found to be similar in structure and function to their mammalian counterparts. C3 of the chicken consists of a two-chain (α and β) structure with a methylamine-sensitive thioester bond, as in mammals. In contrast to mammalian C3, however, chicken C3 exists in three molecular forms, and yet, genetic polymorphism has not been demonstrated (19). Furthermore, its concentration in serum, amounting to approximately 0.5 mg/ml, is about half of that observed in humans.

To expand our knowledge of the chicken complement system, we initiated studies to characterize chicken C3. In this study, we have simplified the purification of chicken C3, obtained cDNA clones encoding this protein, analyzed the conservation of functional sites in the molecule and correlated them to the analogously conserved structural elements, and constructed a phylogenetic tree for C3 and other C3-related proteins. In addition, we present the sequence of the thioester region of two chicken α_2-macroglobulin (α_2M)[3] related proteins and analyze their similarity to chicken C3 and other related proteins.

Materials and Methods

Materials

Chicken serum (from white leghorn, 5 to 7 wk old), obtained after clotting at 4°C for 30 min, and EDTA-plasma were purchased from Cocalico Biologicals, Inc. (Reamstown, PA). All chemicals used for automated sequencing were obtained from Applied Biosystems (Foster City, CA). DNA modification and restriction enzymes were purchased either from Boehringer Mannheim (Indianapolis, IN) or Promega (Madison, WI). All radionucleotides were obtained from DuPont NEN (Boston, MA), and all chemicals and reagents were reagent or higher grade.

Isolation of mRNA

cDNA was synthesized from RNA isolated as described by Maniatis et al. (20). Fresh chicken liver was homogenized in a solution containing 4 M guanidine thiocyanate, 0.1 M Tris-HCl, pH 7.5, 0.14 M 2-ME, and 0.5% sodium laurylsarcosine. The RNA was pelleted through 5.7 M CsCl in an ultracentrifuge at 22,000 rpm for 20 h (SW 24 rotor), dissolved in water, and then precipitated twice with 0.2 M sodium acetate in 70% ethanol.

Poly(A)$^+$ RNA was isolated by oligo(dT) affinity chromatography, using the poly(A) tract-mRNA isolation kit (Promega) according to the manufacturer's instructions.

Reverse transcription-PCR

To isolate chicken C3 cDNA, RT-PCR was conducted in a manner similar to that described by Nonaka and Takahashi (11). Double-stranded cDNA was synthesized from 1 μg of poly(A)$^+$ RNA and random hexanucleotides primers using the cDNA synthesis kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Based on the conserved C3 amino acid sequences GCGEQ$^N/_T$M and TWLTA$^Y/_I$V, two degenerate oligonucleotides were designed and used as PCR primers: primer 1, 5′-ACRAANGCNGTNAGCCANGT-3′ (extends downstream), and primer 2, 5′-GGNTGYGGNGARCARAAYATG-3′ (extends upstream), in which R, Y, and N indicate either A or G, either C or T, and A, C, G or T, respectively.

The conditions for the PCR were denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and polymerization at 72°C for 1 min. The reaction was initiated by adding 5 U Taq DNA polymerase (Cetus, Norwalk, CT), after which 35 reaction cycles were conducted. The reaction products were separated by agarose gel electrophoresis, and the ~220-bp PCR product was extracted from low melting point agarose and subcloned into a pCR1000 plasmid vector using the TA cloning kit (Invitrogen, San Diego, CA). Recombinant plasmid DNA was purified using the QIAGEN kit (QIAGEN Inc., Stusio, CA) according to the procedure recommended by the supplier.

Screening of cDNA library and DNA sequencing

To obtain chicken C3 cDNA clones, a chicken liver 5′-stretch λgt10 cDNA library constructed with methylmercuric hydroxide mRNA (Clontech Laboratories, Inc., Palo Alto, CA) was screened using DNA probes labeled with [α-^{32}P]dCTP by the random primed oligolabeling method (Boehringer Mannheim random-primed DNA labeling kit). Approximately 10^6 phages were screened by plaque hybridization according to the method of Benton and Davis (21). Prehybridization and hybridization were done in 0.5 M Na_2HPO_4/NaH_2PO_4, pH 7.2, with 7% SDS and 1% BSA at 67 to 70°C for 2 and 16 h, respectively. The filters were washed three times in 40 mM Na_2HPO_4/NaH_2PO_4, pH 7.2, with 5% SDS and 1 mM EDTA at 60°C for 20 min per wash.

Positive clones were analyzed by PCR and Southern blotting. Clones containing a C3 insert were grown up on agar plates, and phage DNA was isolated using the QIAGEN λ-phage midi kit. Inserts were isolated by *Eco*RI digestion or PCR and were subcloned into pIBI-31 (IBI, New Haven, CT) or pCR228II (Invitrogen) plasmid vectors, respectively. DNA sequencing of both strands was performed according to Sanger et al. (22) using the Sequenase sequencing kit (USB, Cleveland, OH); on average, each strand was sequenced 1.4 times. Templates for DNA sequencing were alkali-denaturated recombinant plasmids. All the oligonucleotides were synthesized using an automated DNA synthesizer (Cyclone Plus; Millipore, Bedford, MA).

Preparation of anti-C3 Abs

A 21-amino acid peptide C3$^{741-761}$ (SEVDDAFLSDEDITSRSLFPE) representing the amino terminus of the chicken C3 α'-chain (Fig. 2) was synthesized using an Applied Biosystems 430A DNA synthesizer as described (23). The purity of the peptide was assessed by HPLC and its mass confirmed by laser desorption mass spectroscopy (24). The peptide was coupled to keyhole limpet hemocyanin (KLH) using the glutaraldehyde method (25), and an Ab against it (anti-chicken C3$^{741-761}$) was raised in rabbits.

Purification of chicken C3

Chicken C3 was purified by a modification of the method of Alsenz et al. (4). Chicken plasma treated with 2 mM PMSF, 50 μM leupeptin, 5 mM EDTA, and 10 mM EACA was brought to 13% (w/v) polyethylene glycol (PEG), with constant stirring at 4°C for 20 min. The plasma was then

[3] Abbreviations used in this paper: α_2M, α_2-macroglobulin; RT-PCR, reverse transcription-PCR; PZP, pregnancy zone protein; KLH, keyhole limpet hemocyanin; PEG, polyethylene glycol.

centrifuged at 10,000 × *g* for 20 min. The pellet was redissolved in 5 mM phosphate buffer, pH 7.5, containing all the inhibitors listed above and applied to a 5 × 8 cm DEAE HR-40 (Millipore) anion exchange AP-5 column. Chicken C3 was eluted with a linear gradient of NaCl (0 to 750 mM). Fractions containing C3 were identified by Western blotting using the rabbit anti-chicken $C3^{741-761}$ Ab and by their mobility in 7.5% SDS-PAGE gels (26). The reactive fractions were pooled and the sample was adjusted to pH 5.8 and conductivity of 9 mS (at 25°C) by the sequential addition of 100 mM HCl and H_2O. The sample was applied to a Mono S 5/5 cation exchange column (Pharmacia Biotech Inc., Piscataway, NJ) equilibrated in 10 mM sodium phosphate buffer, pH 5.8, with 100 mM NaCl (9 mS at 25°C) and a gradient up to 500 mM NaCl was developed. All the chromatographic separations described above were performed using an HPLC system (Waters 650 E Advanced Protein Purification System). To determine whether the purified C3 contains an intact thioester bond, it was iodinated and added to chicken serum, which was subsequently activated by zymosan. The serum was incubated with zymosan (5 mg/ml serum) for 30 min at 37°C in the presence or absence of EDTA. The zymosan particles were boiled in 2% SDS/500 mM NaCl and extensively washed with the same solution. The amount of zymosan-bound iodinated C3b/iC3b was determined by measuring the zymosan-associated radioactivity in a gamma counter.

Determination of NH_2-terminal amino acid sequence

The NH_2-terminal amino acid sequence of chicken C3 was obtained by sequencing the α- and β-chains of C3 and C3 fragments generated by activating the chicken serum with zymosan or by extensive tryptic digestion.

To obtain the NH_2-terminal sequence of the C3 chains, the intact molecule was reduced, subjected to electrophoresis, and electroblotted onto ProBlott membranes (Applied Biosystems) (23). The internal protein sequence for chicken C3 was obtained by digesting it with the endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer Mannheim). Digestion was conducted at a C3:enzyme ratio of 100:1 (w/w) in PBS, pH 7.5, with 0.1% SDS and 5 mM DTT for 16 h at 22°C. The SDS was precipitated in 1 M guanidine HCl and the proteolytic mixture was injected onto a microbore reversed-phase C4 column (2.1 × 220 mm) equilibrated with 0.1% trifluoroacetic acid. The separation was performed using the Applied Biosystems Micro Separation system 130A at 25°C and a flow rate of 200 μl/min. The C3 peptides were eluted with a 14-ml gradient of 0 to 63% CH_3CN containing 0.1% trifluoroacetic acid and detected at 214 nm; the positive fractions were collected and the masses of the isolated peptides were determined by matrix-assisted laser desorption spectrometry (VG Tofspec; Fisons Pharmaceuticals Ltd., England). Fractions containing single peptides were subjected to gas-phase sequencing.

To obtain the NH_2-terminal sequence of chicken C3 fragments fixed onto zymosan upon complement activation, chicken serum was incubated with zymosan (5 mg/ml) for 60 min at 37°C and the C3 fragments were eluted from zymosan as described (13). The eluted C3 fragments were separated on 10% SDS-polyacrylamide gels, electroblotted, and subjected to automated Edman degradation using an Applied Biosystems 473A protein sequencer.

Computer-assisted sequence analysis

The protein sequences of C3, C4, C5, α_2M, pregnancy zone protein (PZP), murinoglobulin-1 (MUG-1), and α 1 inhibitor III (A1I3) were taken from the Swiss Protein Sequence databases or were translated from the cDNA sequences obtained from GenBank. The sequence around the thioester site of chicken ovostatin was abstracted from Reference 27. The sequence analyses were made using the PcGene (IntelliGenetics Inc., Mountain View, CA), GCG (University of Wisconsin, Madison, WI). Phylogenetic trees based on protein sequences were constructed using three different methods: the protein sequence parsimony method (PROTPARS) from the PHYLIP package (28), the neighbor-joining method (29) from the MEGA package, version 1.02 (30), and the fast approximation alogorithm of Hein (31). Phylogenetic trees based on the nucleotide sequence were constructed with the neighbor-joining method (29) on the basis of nonsynonymous nucleotide differences per site. This analysis included only sequences that correspond to the α-chain of C3 because the β-chain region has a high rate of nonsynonymous nucleotide subsitutions and may provide less reliable information (32). The sequences used in the PROTPARS and neighbor-joining methods were aligned using the PILEUP program of GCG and gaps were removed from the phylogenetic analysis.

Results

Isolation of chicken C3 cDNA clones and nucleotide sequencing

To clone the gene encoding chicken C3 we generated a cDNA probe by RT-PCR using chicken liver mRNA and degenerate primers based on the protein sequences $GCGEQ^N/_TM$ (the thioester site) and $TWLTA^Y/_FV$, which are found to be conserved in C3, C4, and α_2M of different species (see below and Fig. 8) (11, 13). The resulting PCR products showed a major band of ~220 bp, as expected from the sequences of C3 from other species. Subcloning of the ~220-bp product into the PCR1000 plasmid vector yielded five clones. One of the clones (Ch12) showed an 81% amino acid sequence similarity to human C3, and the other four (three of which were identical in sequence) had a 75 to 90% similarity to human α_2M and PZP (see below and Fig. 8).

We then obtained additional clones by screening a chicken liver λgt10 cDNA library with the Ch12 insert obtained by PCR. We isolated seven clones, the longest of which (clone 4.1.1) was 4.1 kb (Fig. 1). This clone was sequenced by digesting it with *Eco*RI and subcloning it into a pIBI-31 plasmid vector. The insert of the obtained plasmid, pIBI-31–4.1.1, was further digested with *Pst*I and the three largest fragments were subcloned into the pIBI-31 vector to facilitate sequencing. The compiled sequence contained an open reading frame of 3743 bp and a 300-bp untranslated trailer sequence at the 3′ end. To isolate clones representing the 5′ end of chicken C3 mRNA, we screened the library with an 874-bp PCR fragment overlapping the 5′ end of the 4.1.1 clone (bases 1506–2380; probe 1 in Fig. 1). This screening yielded a 958-bp clone (clone 24.5.1) that overlapped the sequence of clone 4.1.1 by 400 bp and extended another 558 bp toward the 5′ end. Further screening, with a 249-bp PCR fragment from the 5′ end of clone 24.5.1 (probe 2, Fig. 1) produced two clones (34.1.1.1 and 35.1.1) that cover the 5′ end of chicken C3 cDNA and extend 300 and 70 bp, respectively into the untranslated leader sequence (Fig. 1). In all, our screening of 10^6 clones yielded 35 C3-positive clones. The compiled chicken C3 mRNA (Fig. 2) contains an open reading frame of 4956 bp, followed by a 300-bp 3′-untranslated region that includes a $poly(A)^+$ tail of at least 50 nucleotides. The sequence found at the proposed start site (*underlined*) of C3 translation, **GCCATGG** with G (*boldface*) at positions −3 and +4, agrees with the consensus sequence found in other vertebrate genes, thus suggesting that the C3 translation starts at this position (33). The deduced amino acid sequence contains 1653 residues, with 18 (based on the "(−3,−1)-rule") representing the putative signal peptide (34), 640 the β-chain, and 989 the α-chain. The processing signal for the generation of the

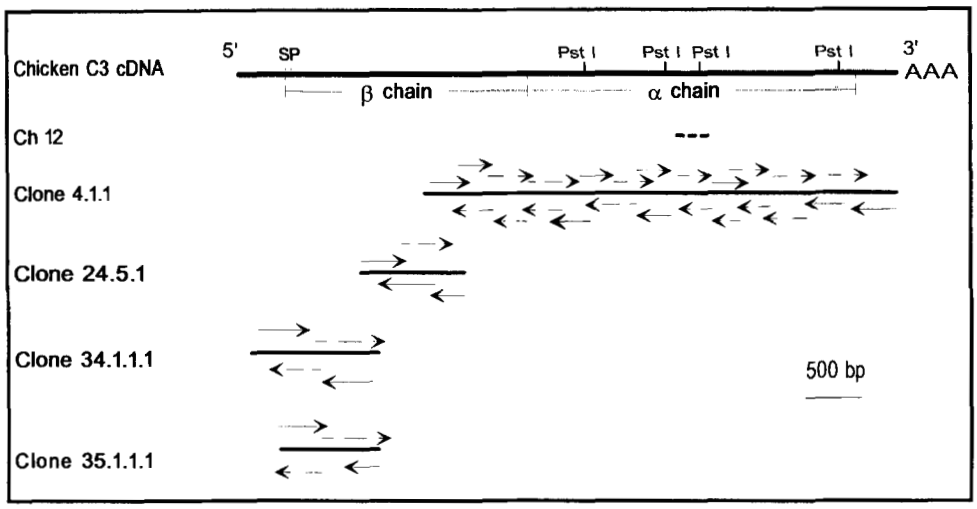
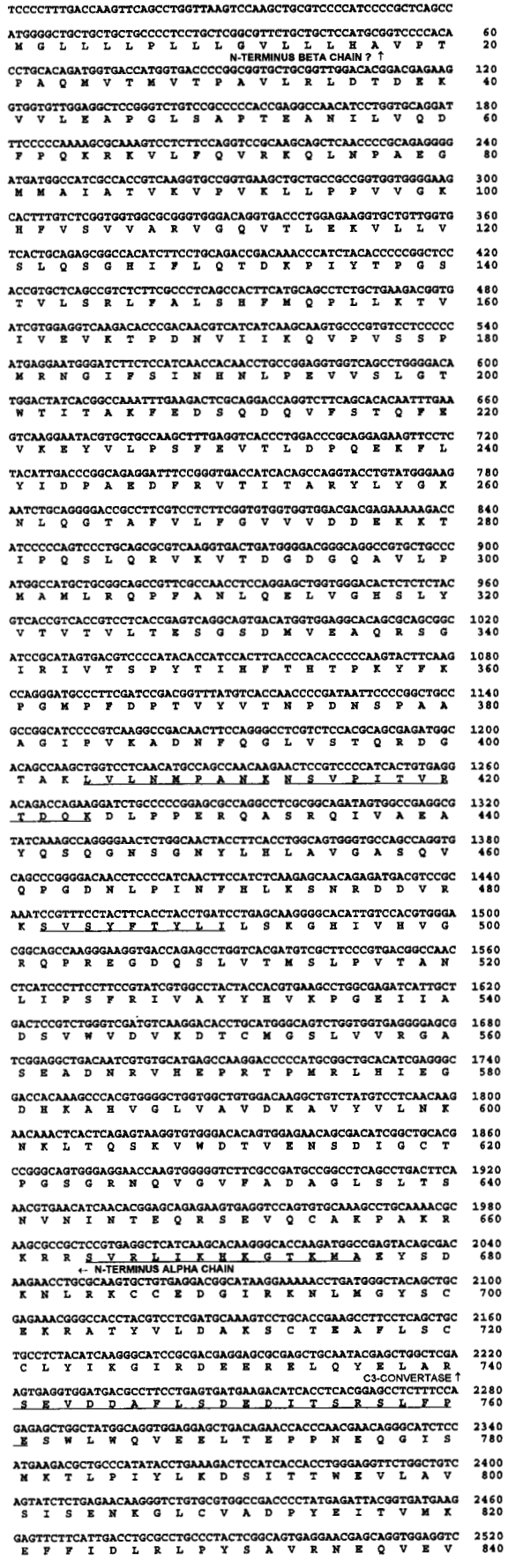


FIGURE 1. Map of clones and sequencing strategy used for the sequencing of chicken C3. A schematic drawing of chicken cDNA, the α- and β-chains, the signal peptide (SP), the relative position of C3 clones, and the *PstI* restriction sites of clone 4.1.1 are indicated. Solid arrows represent sequencing of clones using a universal priming site in the cloning vector, whereas dotted arrows represent sequences determined through the use of a series of consecutive oligonucleotides, each of which was synthesized based on the sequence determined for the end of the preceding segment of the inserted DNA. The dotted line represents the position of PCR probes used to screen the cDNA library.

two-chain molecule is located in the same position as in other species, but is an RKRR sequence instead of the RRRR of most other species (13). The calculated molecular mass of 183,397 Da includes a 111,190-Da α-chain and a 70,340-Da β-chain. There are three potential *N*-glycosylation sites in the α-chain, all of which are glycosylated (Lambris et al., manuscript in preparation). The predicted isoelectric point of 6.67 is close to the 6.4 to 6.6 reported by Laursen and Koch (19). The chicken C3 sequence contains 27 Cys residues in the same positions as in mammalian C3s. The amino acid sequence identity of the chicken C3 to that of human, mouse, and rat C3 is 54%; to guinea pig, rabbit, cobra, *Xenopus*, trout, hagfish, and lamprey C3 it is 53%, 52%, 57%, 55%, 44%, 31%, and 33%, respectively. In comparison, its sequence identity to human C4, C5, and α_2M is significantly lower (23 to 31%). We constructed a phylogenetic tree for C3, C4, C5, and members of the α_2M family by first aligning the sequences on the basis of maximum amino acid similarity. The phylogenetic tree shown in Figure 3 was constructed using the neighbor-joining method with Poisson corrections for calculation of the distance matrix. Although the bootstrapping value of some branches was supported in only 60 to 68% of the bootstrap trees, this topology is in agreement with that obtained using the protein parsimony and the fast approximation algorithm methods (data not shown). The same topology was also obtained using distance matrixes calculated using *p*-distances or proportion of different amino acids.

Purification of chicken C3 and sequence confirmation by peptide analysis

To confirm that the obtained cDNA sequence represents that of chicken C3 and to characterize processing sites of chicken pro-C3, C3 was purified from chicken plasma. Although it involved five chromatographic steps, the previously published method for the purification of chicken C3 did not yield homogeneous preparations, as the end product contained a 73-kDa contaminating protein (19). We have now simplified the purification of chicken C3 and have obtained highly purified C3 after PEG precipitation and chromatography on an anion and a cation exchange column (Fig. 4). To detect C3 in chromatographic samples, we generated an anti-chicken C3-specific Ab by immunizing rabbits with a KLH-conjugated synthetic peptide, $C3^{741-761}$, representing the first 21 residues of the α′-chain of chicken C3. We selected this segment on the basis of our earlier observation that Abs to the corresponding segment of human C3 are reactive with C3 and C3 fragments (C3b/iC3b/C3c) in Western blotting and direct binding ELISA (35). The generated Ab, similar to that against human C3 peptide, recognized C3 bound to ELISA plates and in Western blots. The total recovery of purified C3 from 5 ml plasma was 1.7 mg. To determine whether the purifed C3 contains an intact thioester bond, it was iodinated and added to chicken serum that was subsequently activated by zymosan. Five percent of the added C3 was fixed onto zymosan (data not shown), which suggests that most of the purified C3 is active; during complement activation, only 5 to 10% of the activated C3b gets fixed onto zymosan. To determine whether chicken C3 is indeed cleaved at the site we predicted by analogy to other C3 sequences, we obtained the NH_2-terminal amino acid sequence of the α′-chain of chicken C3 by sequencing the chicken C3 fragments eluted from zymosan (see *Materials and Methods*). The NH_2-terminus of this fragment (Fig. 2) starts at Ser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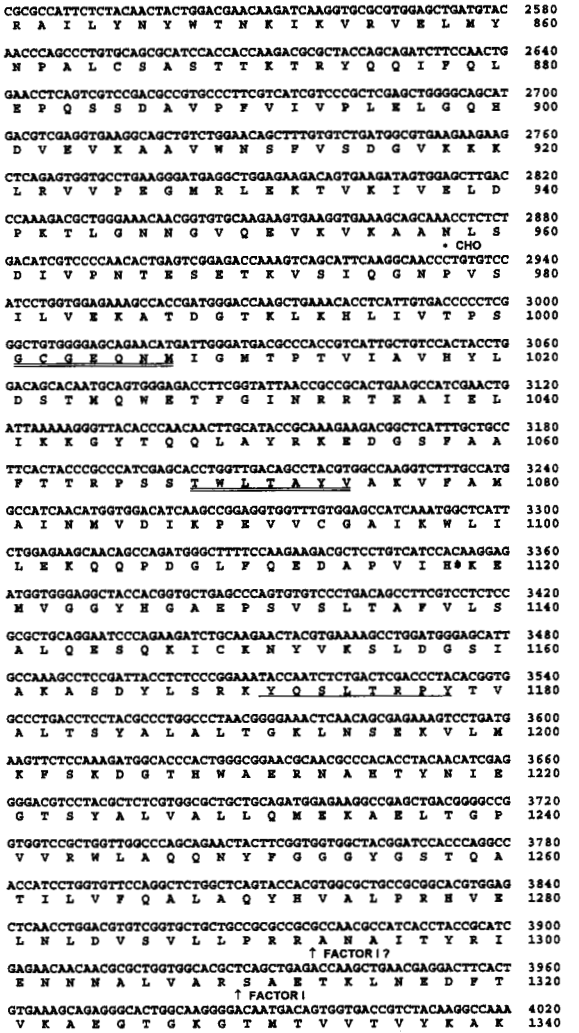
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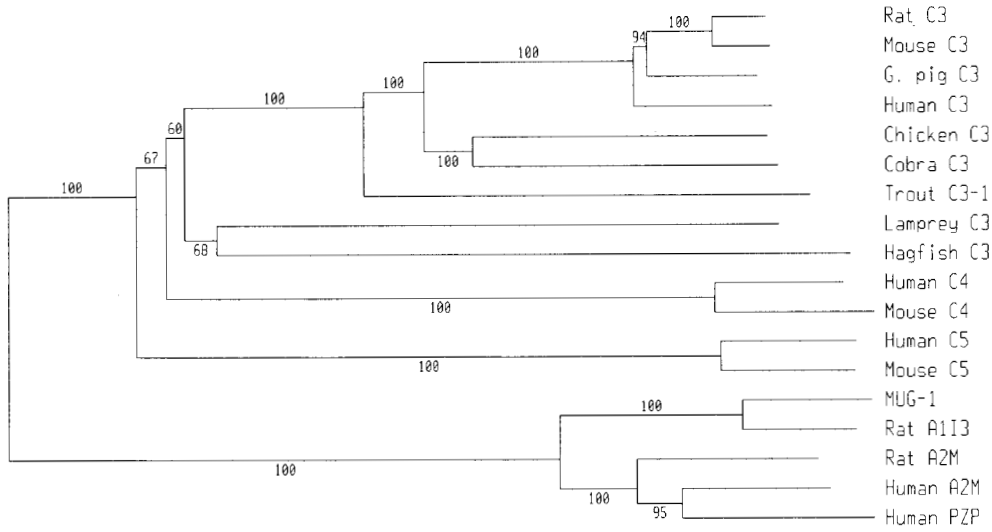

FIGURE 3. Phylogenetic relationships of C3s and other homologous proteins. The tree was constructed using the neighbor-joining method of Saitou and Nei (29). Sequences were aligned using the PILEUP program of the GCG package and gaps were excluded from phylogenetic analysis. The distance matrix was obtained using Poisson corrections and the degree of support for internal branches was assessed using the bootstrapping method with 1000 bootstrap replications. The rabbit and *Xenopus* C3 sequences were not included in this analysis, as their complete sequences are not available. Numbers on branches are bootstrap percentages supporting a given branch.

741, indicating that chicken C3 convertase does indeed cleave the Arg^{740}-Ser^{741} bond. The Ab we generated is monospecific by Western blotting (Fig. 5) and recognizes C3 fixed to ELISA plates. The Ab reacts with a 180-kDa protein under nonreducing conditions and with a 111-kDa (α-chain) protein under reducing conditions (Fig. 5). Analysis of several commercial chicken sera by Western blotting showed that the C3 in most sera is in the iC3b form. In contrast, when we obtained serum by clotting the blood at 4°C for 30 min and then storing it immediately at −70°C, we did not observe significant degradation to iC3b; we therefore recommend this collection and storage procedure when chicken serum is to be used for hemolytic assays.

The isolated chicken C3 migrates in SDS-PAGE as a 180-kDa protein, which upon reduction gives rise to two bands of 116 kDa and 67 kDa (Fig. 5). These m.w. estimates are close to those calculated from the deduced protein sequence when taking into account the glycosylation of the α-chain. When the individual chains of C3 were separated by SDS-PAGE and subjected to Edman degradation, we were able to obtain a sequence only for the α-chain. This sequence starts at Ser 664 (Fig. 2), thus confirming that pro-C3 is indeed cleaved at the RKRR site to generate C3. All attempts to sequence the β-chain of chicken C3 were unsuccessful, leading us to believe that it is blocked. A blocked N-terminus was confirmed by sequencing the intact C3 molecule, which produced a single α-chain sequence and not the expected α/β sequence.

To confirm that the isolated cDNA clones represent the sequence of the purified C3 protein, we digested C3 with Lys-C, purified the resulting fragments by HPLC, and selected fragments were subjected to spectrometric analysis. The calculated and obtained mass of eight peptides as well as the N-terminus sequence of four peptides sequenced is shown in Table I. The masses obtained for all eight peptides matched the theoretical masses of the Lys-C-generated fragments (Table I). NH_2-terminal sequences of the four peptides and the mass spectrometric analysis were also in agreement. Taken together, these results demonstrate that the isolated protein is encoded by the cDNA sequence presented here.

To confirm that chicken factor I and C3 convertase cleave chicken C3 in the sites predicted from the cDNA sequence, we generated C3 fragments by activating chicken serum with zymosan (zymosan-C3b/iC3b; Fig. 6), analyzed them by SDS-PAGE, and identified their NH_2-terminal amino acids. Electrophoretic analysis of the C3 showed a similar degradation pattern to that observed for human C3 (Fig. 6). Although similar fragments were

FIGURE 2. cDNA and derived amino acid sequence of chicken C3. Underlined amino acids indicate sequences that have been confirmed by Edman degradation. The C3 convertase and the factor I cleavage sites are indicated by (↑) and the potential *N*-linked glycosylation sites by (* CHO). The factor I (?) site is not confirmed by protein sequencing. The sequences from which the degenerate oligonucleotides for the RT-PCR were designed are double underlined.

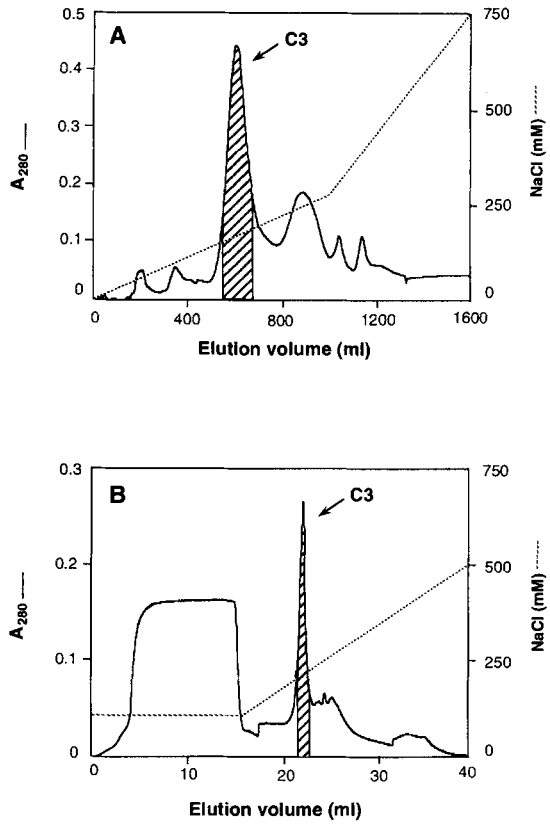

FIGURE 4. Purification of chicken C3. (*A*) The 13% PEG pellet of chicken plasma was resuspended in 5 mM sodium phosphate, pH 7.5, containing 5 mM EDTA and applied to a DEAE HR-40 anion exchange column (5 × 8 cm). Bound proteins were eluted with an NaCl gradient (0 to 750 mM) at a flow rate of 10 ml/min. (*B*) The chicken C3-containing fractions (arrow) from the DEAE HR-40 column were pooled, concentrated, and applied to a Mono S 5/5 FPLC cation exchange column equilibrated in 10 mM sodium phosphate buffer, pH 5.8, with 100 mM NaCl. Bound proteins were eluted with an NaCl gradient (0 to 500 mM NaCl) at a flow rate of 1 ml/min.

found in the presence or absence of EGTA, which inhibits classical pathway, no fragments were detected when the sera were activated in the presence of EDTA, which inhibits both complement pathways. These data suggest that the chicken has proteins with functions analogous to those of the human factors B, D, I, and H. The NH_2-terminal amino acid sequences of the 68-kDa and 43-kDa fragments (Fig. 6) showed that these fragments are the analogues of human C3 fragments generated by C3 convertase and factor I (I_2) (Fig. 7). In addition, these data show that the cleavage site for the C3 convertase is conserved in the C3s of all species except in lamprey C3, in which the cleavage site is Arg-Asn rather than Arg-Ser; the factor I (I_2) cleavage site is Arg-Ser in mammalian and chicken C3 and Arg-Thr in cobra and trout C3.

Partial cDNA sequence of two chicken proteins of the α_2M family

Cloning of the RT-PCR product (see above) led to the isolation of five clones. The five inserts were sequenced, and four (Ch7, Ch14, Ch9, and Ch8) showed high sequence similarity to α_2M and related proteins (Fig. 8). The identity of the clone Ch7 sequence to that of other homologous proteins was found to range from 30% to 84% (Table II), with the highest identity being with chicken ovostatin (84%), human α_2M (82%), and PZP (80%). The sequence identity of Ch7 to chicken ovostatin was only 84%, a figure that could conceivably be raised to 96% if it is assumed that there are errors in the protein sequence. However, the fact that chicken ovostatin does not contain a thioester site (27) and is not synthesized by the tissue (liver) from which the library was made (36) excludes the possibility that the Ch7 sequence represents the sequence of chicken ovostatin. The high degree of sequence identity/similarity to human α_2M (82%/87%) and PZP (80%/90%) suggests that this sequence could represent either one of these two proteins, and it would be difficult to determine which. Three of the other clones (Ch14, Ch9, and Ch8) are identical, and their sequence identity to that of α_2M from three different species ranges from 62% to 70%. The similarity of these clones to PZP, AI13, chicken C3, chicken ovostatin, and Ch7 is 68%, 62%, 36%, 58%, and 65%, respectively. The high degree of sequence similarity to the α_2M-related proteins from other species and the relatively lower similarity to the Ch7 sequence suggests that these clones encode a novel α_2M-related protein.

Comparison of the thioester region of C3, C4, C5, and α_2M (and of the novel α_2M protein that we identified in this study) indicates that there are two regions of strong sequence conservation (Fig. 8). Region A includes the thioester site and is conserved in all proteins except C5 and chicken ovostatin. Region B, which includes the sequence that we used to construct the antisense primer for the RT-PCR, is also conserved in all of the proteins. Whether this region is associated with the thioester regions is unclear. Its uniform conservation, however, suggests that it is involved in the function of the thioesters or constitutes an important element of their structure. The high conservation of A and B regions in all proteins suggests that these sequences could be used to isolate clones from other species, including invertebrates.

Discussion

Although aves represent an important step of complement evolution of vertebrates, only limited information is available on the structure and functions of the proteins that comprise it. Chicken C3 has the same chain structure as most other vertebrate C3, and it exists in three molecular forms which appear not to be the result of genetic polymorphism. The primary structure of chicken C3 as well as

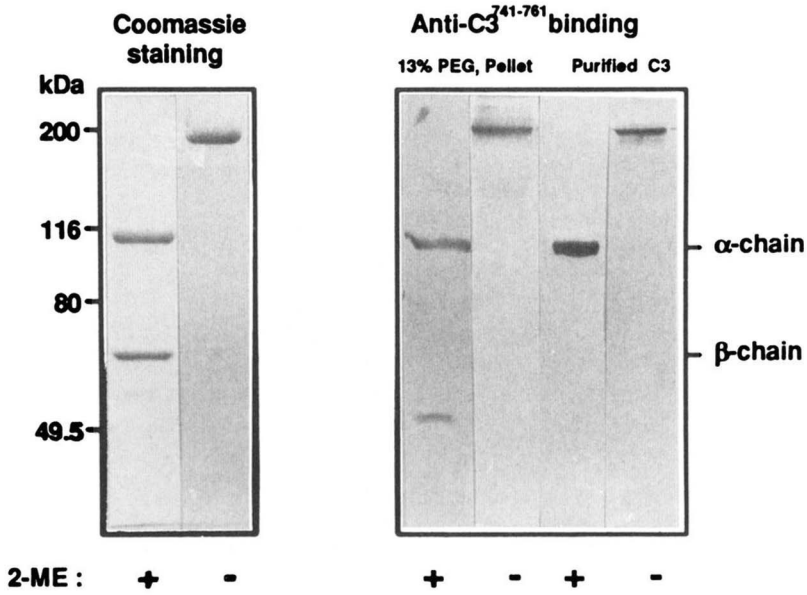

FIGURE 5. SDS-PAGE and immunoblotting of purified chicken C3. Purified chicken C3 and the 13% PEG pellet of chicken plasma were subjected to electrophoresis on a 7.5% SDS polyacrylamide gel under reducing (2-ME: +) or nonreducing conditions (2-ME: −). Gels were stained with Coomassie blue or electrotransferred to polyvinylidene difluoride membranes for immunostaining with anti-$C3^{741-762}$, followed by a horseradish peroxidase-conjugated goat anti-rabbit Ab.

Table I. *Mass spectrometric analysis and NH_2-terminal sequence of selected Lys-C-generated peptides from chicken C3*

Peptide	Position	Calculated Mass	Observed Mass	NH_2-Terminal Sequence
1	481–492	1421	1420	SVSYFTYL
2	1170–1191	2418	2419	YQSLTRPY
3	675–681	843	842.5	ND[a]
4	1538–1599	2461	2460	ND
5	1120–1147	2936	2936.4	EMVGGYHGAEPSV
6	117–133	1898	1898	ND
7	412–423	1358	1357	LVLNMPANK
8	403–411	999	1001	NSVPITVRTDQK

[a] ND, not determined.

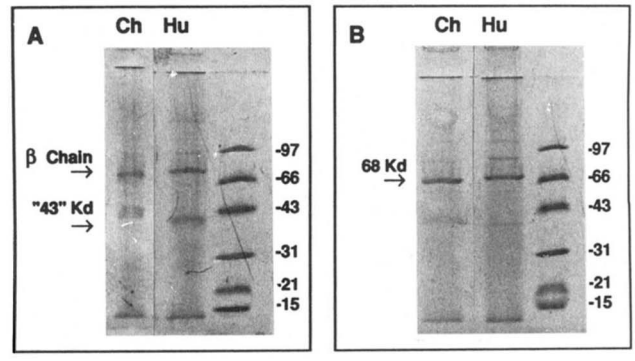

FIGURE 6. SDS-PAGE electrophoresis of chicken and human C3 fragments eluted from zymosan. The zymosan C3 fragments were prepared as described in *Materials and Methods*. (*A*) Fragments eluted after reduction of zymosan-iC3b with 2-ME. (*B*) Fragments eluted after treatment of zymosan-iC3b with hydrazine (pre-eluted as in *A*).

its role in complement activation are unknown. In this report, we present the complete primary structure of chicken C3 and compare it to those of C3 from other species and of other related proteins. We also demonstrate that it is possible to purify chicken C3 to homogeneity by using only two chromatographic steps instead of the five used previously, and present evidence for the presence of factors H-, D-, and I-like proteins in chicken serum. Finally, we present the sequence of the thioester region for two chicken α_2M-related proteins and correlate this sequence to those of C3 and other related proteins.

Several lines of evidence indicate that the DNA sequence we obtained is that of chicken C3: 1) the deduced protein sequence completely matches the partial protein sequence of seven different fragments of chicken C3; and 2) the DNA sequence shows high similarity to those of C3s from other species. Examination of the deduced amino acid sequence of chicken C3 indicates that the molecule is synthesized as a one-chain molecule, like other C3s, and is cleaved at an RKRR sequence, perhaps by a furin-like enzyme (37). Chicken C3 contains three putative *N*-glycosylation sites on the α-chain (N^{958}, N^{1429}, and N^{1570}), all of which are glycosylated (Lambris et al., manuscript in preparation). The conservation of $Arg^{740}Ser^{741}$ and $Arg^{1309}Ser^{1310}$ in the α-chain of chicken C3 suggests that chicken C3 convertase and factor I have specificities similar to those of human counterparts. The cleavage of C3 at the above sites was confirmed by sequencing the zymosan-eluted α'-chain and 40-kDa C-terminal fragment (Fig. 2). The actual cleavage of C3 at Arg^{1292}-Ala^{1293}, as well as the generation of C3c and

	C3 CONVERTASE			K/I?	I_3?	I?		I_1? CLEAVAGE SITES I_2	
	737			931				1291	
	↓			↓	↓	↓		↓ C3f	↓
CHICKEN C3	ELARS	EVDDA...FL	//	EKTV	KIVELDPKTL	GNNGVQEVKV	KAA	// RRAN	AITYRIENNN ALVARSAETK
HUMAN C3	G****	NL*ED...II	//	N***	AVRT***ER*	*RE***KEDI	PP*	// S*SS	K**H**HWES *SLL**E***
RABIT C3				N***	AVRT***EN*	*QG***KEEI	PS*	// S*SS	PVKH**VWDS *SLL**E***
RAT C3	G****	D**ED...II	//	N***	AVRT***EH*	NQG***RED*	N**	// S*SS	PTVF*LLWES GSLL**E***
MOUSE C3	G****	*LEED...II	//	N***	A*HT***EK*	*QG***KVD*	P**	// S*SS	*T*F*LLWE* GNLL**E***
G.PIG C3	G****	DM*ED...II	//	N***	A*RT*N*EQ*	*QG***REEI	P**	// S*SS	PSKF*LVWEA GSLL**EA**
COBRA C3	F****	DFE*E...LF	//	KNI*	T*I****SVK	*VG*T**LT*	I*N	// E*EV	PER*S*NDR* *VQ**TV***
TROUT C3	.*S**	*E**DDDAYM	//	K*E*	NVLL...NPV	KHG*E*TSHI	PSG	// G**S	VTKWS*N*K* QFHT*TDKVN
HAGFISH C3	D*G**	QGE*..F.MI	//	*MS.	..RSWSVQPR	RHG*Q*VIV*	DNE	// ENGV	FDKEFQIT*D NAFVQKPFKV
LAMPREY C3	V*R*N	DFME..LDLM	//	IRS.	..ESRSVHV.	..EERETFFI	*NE	// KNN.	FEKKMKITEE TRFVQEPHKI

FIGURE 7. Amino acid sequence comparison of C3 convertase and factor I cleavage sites in C3s from chicken and other species. Numbering is from the chicken sequence. Asterisks indicate identical residues and periods indicate gaps introduced for maximal sequence alignment. The factor I (I, I_1, I_2, I_3,), kallikrein, and convertase cleavage sites are shown by arrows. (?) indicates putative factor I cleavage sites.

CHICKEN OVO	*N*****VLF**N	IYVLD**DK*	R*LSE.....	.DMKSKTIGY	LVS**QK**S	*KH				
Ch 14	*******VQF**N	IFVLQ**KK*	K*LDP.....	.*IEVIALYF	LRT**QR**L	*KHd*G**S*	*GK..SDTQGN*******			
Ch 7	*******VLF**N	IYVLD**NK*	G*LSE.....	.*VKSK*IGY	LVS**QR*MN	*KHP*G**ST	*GP*Y.RQPGN*******			
MOUSE α_2M	*Y********L***N	*YVLK**NE*	Q*LTQ.....	.KIKTK*LG*	LRA**QRE*N	*KHK*G**S*	*GDQNGEREGN*******			
RAT A1I3	*Y********L***N	*YVLK**NE*	Q*LTE.....	.KIKSK*LG*	LRA**QRE*N	*KHK*G**S*	*GDHNGQGQGN*******			
HUMAN α_2M	*Y********L***N	*YVLD**NE*	Q*LTP.....	.*VKSK*IG*	LNT**QR**N	*KHY*G**ST	*QE*YGRNQGN*******			
RAT α_2M	*Y********L***N	*YVLD**NE*	Q*LTQ.....	.*IKTK*IA*	LNT**QR**N	*KHR*G**S*	*GDKPGRNHAN*******			
HUMAN PZP	*Y********L***N	*YVLN**NE*	Q*LTQ.....	.*IKAK*VG*	LIT**QR**N	*KHQ*G**ST	*GE*YGRNQGN*******			
HUMAN C3	*S********G*T*T	*IAVH***E*	E**EKFG...	L*K*QG*LE*	*KK**TQ**A	*RQPSSA***	*VK*....AP********			
RAT C3	*S********G*T*T	*IAVH***Q*	E**EKFG...	L*K*QE*LE*	*KK**TQ**A	*KQPISA***	*NN*....PP******MW			
MOUSE C3	*A********G*T*T	*IAVH***Q*	E**EKFG...	I*K*QE*LE*	*KK**TQ**A	*KQPSSA***	*NN*....PP********			
RABIT C3	GS********A*THT	*IAVH***H*	E**DKFS...	L*K*QE*LE*	*KK**TQ**A	*KQPNSA***	*LN*....AP********			
G.PIG C3	*S********G*T*T	*IAVH***Q*	E**EKFG...	L*K*QE*LN*	*NR**TQ**A	*KQPNWA***	*KN*....AS********			
CHICKEN C3	*S********G*T*T	*IAVH***S*	M**ETFG...	INR*TE*IE*	*KK**TQ**A	*RKE*G****	*TT*....PS********			
COBRA C3	*S********T*T*S	*IATY***A*	G**ENLG...	VDR*TE*IKQ	*MT**AQ**V	*KKA*H****	*TN*....AS*S******			
XENOPUS C3	*A********STT*S	*IATR***AS	G**ERVG...	VNR*DQ*LKN	MRQ**AQ**A	*RKP*N****	*KD*....PA*****G**			
TROUT C3	*V********Y*TLP	*IATH***N*	KK*EDIG...	LDK*NT*IK*	*NI**QR**A	*RKE*G****	*VS*....QS********			
LAMPREY C3	*T********K***T	TLTLI***SV	QE*EKIG...	LHR*EE*IG*	LKQ**SRE*S	*RKA*H****	*IK*....PS********			
HAGFISH C3	*R*******MYTSIT	*MVAR**NRS	D**NKMGDPQ	L..KKRSFD*	*TS**AS**T	*RKP*Y****	*LH*....AS********			
HUMAN C4	*R*****T**Y***T	LAASR***K*	E**STLPP..	.*TKDH*VD*	*QK**MRIQQ	*RKA*G****	*LS*....DS********			
MOUSE C5	*K*SA*AE*MSI**V	FYVFH***AG	NH*NIFYPDT	LSK*QSLEKK	*KQ*VVSV*S	*RNA*Y**SM	*KGA....SA*******A			
CONSENSUS	P-GCGEQNMI-MAP-	V----YLD-T	-QW-------	-E-R--A--L	I--GY--QL-	Y---D-SYAA	F--R------STWLTAFV			
	A									B

FIGURE 8. Deduced amino acid sequence of chicken Ch7 and Ch14 cDNA clones and comparison to sequence of C3, C4, C5, α_2M, and other related proteins. Sequences identical to the consensus sequence are indicated by stars, and periods denote insertion of gaps. The consensus sequence was made with plurality 11. The numbering is based on the chicken C3 sequence. Double underlined sequences indicate the position of RT-PCR primers.

C3dg, was not determined; of interest, however, is the fact (based on cDNA sequencing) that Arg-Ala is found instead of Arg-Ser at the first factor I (I_1) cleavage site of chicken C3 (residues 1292–1293). At present, it is not known whether chicken factor I cleaves chicken C3b at Arg-Ala, whether C3b is cleaved at a different site before it is cleaved at residues 1309–1310, or whether the cleavage starts at residues 1309–1310. At the third factor I cleavage, an Asn-Asn is found, based on our alignment using the PILEUP software, instead of the Arg-Glu that is found in the human C3 (Fig. 7). Although no Arg-Ser/Thr factor I cleavage sites were found near the Asn-Asn bond, two potential cleavage sites were found that include Lys^{932}-Thr^{933} and $Lys^{942}Thr^{943}$. If indeed chicken C3b is cleaved to C3c and C3dg, then, either this cleavage is mediated by an enzyme other than factor I or chicken factor I has specificity for Lys-Thr bonds. In either case, the cleavage of C3b could start in any of the above sites. In support of the view that the cleavage of C3b to C3c and C3dg could start in sites other than the Arg-Glu (as in human C3) are the following findings: 1) a Gln instead of an Arg is found in all mammalian C3s at the third position (I_3; Fig. 7) and mutation of Arg-Ser to Gln-Ser at residues 1298–1299 of human C3 (1309–1310 of chicken C3) makes C3 resistant to cleavage by factor I (38); and 2) Ekdahl et al. (39) have identified three C3dg-like fragments with their NH_2-termini starting at residues 933 (cleavage between Arg-Glu), 939

Table II. *Amino acid sequence conservation between RT-PCR-isolated chicken clones and other thioester-containing proteins*

Other Proteins	% Identity/Similarity[b]			
	Ch ovo[a]	Ch14	Ch7	Ch12
CHICKEN ovo	100	58/76	84/94	36/56
Ch14		100	65/75	36/58
Ch7			100	28/39
Ch12				100
MOUSE MUG-1	68/84	70/74	70/81	27/39
RAT A1I3	70/82	62/71	76/84	24/35
HUMAN α_2M	74/86	68/75	82/87	28/40
RAT α_2M	70/84	67/71	76/87	28/40
HUMAN PZP	68/86	68/75	80/90	27/38
HUMAN C3	32/56	32/54	31/42	73/81
HUMAN C4	34/52	39/55	30/38	53/68

[a] ovo, ovostatin; α_2M, α_2-macroglobulin; A1I3, α 1 inhibitor III; PZP, pregnancy zone protein; MUG-1 murinoglobulin 1.

[b] Amino acids considered to be similar are A, S, T; D, E; N, Q; R, K; I, L, M, V; F, Y, W.

(cleavage between Lys-Glu), and 919, 924, or 930 (cleavage between Lys-Thr, Arg-Thr, or Arg-Leu). The nature of the enzyme mediating some of these cleavages is still unclear.

The purification of chicken C3 was accomplished by using only two chromatographic steps, in contrast to five used in previously published purifications. The purified C3 was more than 95% pure as determined by SDS-PAGE and did not contain any visible Coomassie blue-stained contaminants. The purification protocol published earlier by Laursen and Koch (19) consists of five chromatographic steps, and the final product contains a contaminating protein that migrates under reducing conditions as a 73-kDa protein. We observed a similar contaminating protein after the anion exchange column and identified it by NH_2-terminal sequencing as IgG (40). We observed that chicken IgG when subjected to electrophoresis under nonreducing conditions migrates to the same place as C3, and its light chain under reducing conditions is not stained well by Coomassie blue. Chicken IgG has been shown to have mannose-binding activity, and it is conceivable that it reacts with chicken C3 *N*-linked carbohydrates. In human C3, these carbohydrates have been found to be $Man_5GlcNac_2$, $Man_6GlcNac_2$, $Man_8GlcNac_2$, and $Man_9GlcNac_2$; the latter two of which mediate the binding of human C3 to bovine conglutinin (41, 42). The co-purification of chicken IgG with chicken C3 even after five chromatographic steps and two precipitations with PEG (19) suggests an association between these two molecules. If this C3–IgG complex does indeed exist it is susceptible to dissociation by low pH (43), as these two proteins were separated when we subjected the C3-containing fractions to cation exchange chromatography at pH 5.8; all purification steps performed by Laursen and Koch were performed at pH 7.4 to 8.0. Given the relative purity of the C3 we obtained and the ease of purification, the approach we present should facilitate the isolation of chicken C3 for further studies.

After constructing a phylogenetic tree, we found that C3, C4, C5, and α_2M are grouped in four clusters that correlate with the functions of the different proteins of this family (Fig. 3). The relative high pairwise percentage identities between sequence pairs of C3 with other C3-related proteins are consistent with the hypothesis that all of these proteins are derived from a common ancestor. There is a 77 to 80% sequence identity among mammalian C3s, and a 50 to 53%, 52 to 54%, 43 to 45%, and 51 to 54% identity of mammalian C3s as a group to those of cobra, chicken, trout, and *Xenopus*, respectively. The substantially lower identities (28 to 33%) of mammalian C3 to lamprey and hagfish C3 were roughly equal to those observed for C4 (27 to 30%) and C5 (26 to 29%). Our observation that C3, C4, C5, and α_2M proteins segregate into distinct clades is consistent with the occurrence of a duplication event that led to the generation, possibly from an earlier more primitive α_2M-like protein, of a complement protein that is the ancestor of C3, C4, and C5. The phylogenetic analysis reported here suggests that C5 diverged first from this common ancestor and that C3 and C4 diverged later. This hypothesis is in agreement with the conclusion of Hughes (32), who constructed a phylogenetic tree for these genes based on the nucleotide sequence that corresponds to the C3d region of C3. It is, however, in contrast to the hypothesis of Nonaka and Takahashi, who suggested that C4 was the first between C3, C4, and C5 to diverge (11), and also in contrast to the apparent absence of a C5-like protein in cyclostomes (44). When we constructed a tree using the α-chain nucleotide sequences by the neighbor-joining method (data not shown), the obtained phylogenetic relationship for C3, C4, C5, and α_2M is consistent with Nonaka's hypothesis. Thus, no final conclusions regarding the evolution of C3-related proteins can be made until more components are described.

Our data provide additional evidence to suggest that lampreys and hagfishes belong in the same phyla, an assignment that remains under discussion. On the basis of morphologic analysis and fossil data, lampreys are considered to be more closely related to the jawed vertebrates (gnathostomes) than to hagfishes, suggesting a paraphyletic cyclostomata (45). Recent molecular analysis of the small subunit (18S) rRNA sequences from hagfishes, lampreys, chondrichthyan fish, tunicates, and cephalochordates supports the monophyly of the cyclostomes (46). Our observations from the phylogenetic analysis of both protein and nucleotide sequences are in agreement with this analysis.

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