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Multiple forms of complement C3 in trout that differ in binding to complement activators

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ABSTRACT **In all other species analyzed to date, the functionally active form of complement component C3 exists as the product of a single gene. We have now identified and characterized three functional C3 proteins (C3-1, C3-3, and C3-4) in trout that are the products of at least two distinct C3 genes. All three proteins are composed of an α- and a β-chain and contain a thioester bond in the α-chain. However, they differ in their electrophoretic mobility, glycosylation, reactivity with monospecific C3 antibodies, and relative ability to bind to various surfaces (zymosan, *Escherichia coli*, erythrocytes). A comparison of the partial amino acid sequences of the three proteins showed that the amino acid sequence identity/similarity of C3-3 to C3-4 is 87/91%, while that of C3-3 and C3-4 to C3-1 is 51.5/65.5% and 60/73% respectively. Thus, trout possess multiple forms of functional C3 that represent the products of several distinct genes and differ in their ability to bind covalently to various complement activators.**

The complement system plays a key role in host defense against infection (1). Of the 30 distinct complement proteins recognized to date, the third component of complement, C3, is probably the most versatile and multifunctional. This molecule participates directly in the immune surveillance and immune response pathways (2, 3) by interacting with numerous serum, cell-surface, and foreign proteins. C3-like activity has been reported in a variety of species, including invertebrates, and C3 proteins have been identified and sequenced in many different species (4, 5); however, the evolution of this molecule has not been well defined. In all species analyzed thus far, functionally active C3 exists as the product of a single gene (5). Although two different C3-related genes have been identified in the cobra, one in the liver that encodes serum C3 and the other in the venom glands that encodes cobra venom factor (6), it is not clear that the gland gene encodes functionally active C3.

Studies of tetraploid fish have greatly expanded our understanding of the evolution of protein families (7, 8). The trout, a member of this group, has a complement system analogous to that of mammals (9, 10). C3 purified from trout plasma has a structure and properties similar to those of mammalian C3 (11, 12). It consists of two disulfide-linked polypeptide chains, containing a thioester site, and is glycosylated in the β-chain (12). A variant of trout C3, termed C3-2, has been identified that yields a tryptic peptide map significantly different (a 20% mismatch in the peptides) from that of C3-1 (13). The gene encoding trout C3-1 has been cloned, and the protein has been found to have a 30–45% amino acid sequence identity to C3 from other species (14); for C3-2 only limited sequence is available (13).

In this study we have isolated two additional forms of trout C3 (termed C3-3 and C3-4) and have demonstrated that C3-1, C3-3 and C3-4 are the products of at least two, and probably three, separate genes. In addition, we have shown that all these C3 molecules are functionally active; however, they differ markedly in their ability to be fixed to the surface of complement activators.

MATERIALS AND METHODS

Fish. Rainbow trout (*Salmo gairdneri*) were obtained from Landenberg Trout Farm (Philadelphia) and Rheinbrucke (Basel). Blood was collected with a syringe from the caudal artery, and serum was obtained by incubating the blood at 4°C for 4 hr. Trout livers were excised and either frozen in liquid nitrogen or used immediately to prepare the cDNA libraries.

Purification of Trout C3. The various forms of trout C3 (C3-1, C3-3, and C3-4) were purified from trout serum by a modification of the method used to purify trout C3-1 (12). Trout serum (10 ml) was precipitated with 16% polyethylene glycol at 4°C for 30 min in the presence of 20 mM EDTA, 10 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. The precipitate was collected by centrifugation (15,000 $\times$ *g* for 15 min at 4°C), resuspended in 10 mM sodium phosphate buffer, pH 7.4, loaded onto a DEAE 40 HR (6.5 $\times$ 5.0 cm) anion exchange chromatography column (Millipore) previously equilibrated with the same buffer, and eluted at room temperature with a linear salt gradient (0–500 mM NaCl). Purification of the three proteins was monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. A polyclonal anti-C3-1 trout antibody that reacts with the β-chain of C3-1 was used to distinguish C3-1-containing fractions from those containing C3-3 and C3-4. The fractions containing C3-1 were pooled (pool 1), concentrated ten times, diluted in 20 ml of 10 mM sodium phosphate buffer, pH 5.8, and adjusted to pH 5.8. Pool 1 was passed through a Mono S HR 5/5 cation exchange chromatography column (Pharmacia) in 10 mM sodium phosphate buffer, pH 5.8. and eluted as before with 0–500 mM NaCl. The fractions containing C3-3 (pool 2) and C3-4 (pool 3) were pooled, concentrated 10 times, diluted in 10 mM sodium phosphate buffer, pH 6.0, and in 10 mM sodium phosphate buffer, pH 5.8, respectively. Pool 2 was adjusted to pH 6.0 and pool 3 to pH 5.8. Each of these two pools was passed through a Mono S HR 5/5 cation exchange chromatography column equilibrated with 10 mM phosphate buffer at pH 6.0 or pH 5.8 and eluted with 0–500 mM NaCl. Fractions containing C3-3 or C3-4 were pooled, concentrated and subjected to gel filtration on Superose 12 (Pharmacia).

Detection of the Thioester Bond and Concanavalin A-Binding Carbohydrates in the Various Forms of C3. The thioester bond and the concanavalin A-binding carbohydrates in the various C3 molecules were detected as described previously (12).

Preparation and Reactivity of Anti-C3 Antibodies. Antibodies recognizing individual chains of different C3 were generated in rabbits by immunization with SDS-PAGE-purified

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Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U61753).
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chains (14). The reactivity of each form of C3 with the various anti-C3 antibodies was detected by ELISA. Purified protein in 10 mM phosphate-buffered saline (PBS), pH 7.2 (0.1 μg/well), was added to the ELISA plate (Dynatech Laboratories, Chantilly, VA) and incubated at 22°C for 2 hr. Wells were saturated with 2% milk in PBS, pH 7.2 for 30 min at 22°C, washed, and incubated with various dilutions of antiserum for 1 hr at 22°C. Antibodies bound to C3 were detected by incubation with a 1:1000 dilution of peroxidase-labeled goat anti-rabbit IgG (Bio-Rad) for 30 min at 22°C. Color was developed by adding ABTS®substrate (Boehringer Mannheim). Between steps the wells were washed with 0.05% Tween in PBS, pH 7.2. Optical density was measured at 4.5 nm.

Determination of C3 Concentration in Serum. The serum concentrations of the three trout C3 proteins were determined as follows: Various concentrations of purified proteins together with various dilutions of trout serum were run on 7.5% SDS-PAGE and subjected to Western blot analysis. Each C3 was treated with an isoform-specific rabbit polyclonal anti-C3 antibody and peroxidase-labeled goat anti-rabbit IgG (Bio-Rad). After color development the blot was scanned for densitometric analysis. A standard curve was generated for each protein by plotting the concentration of purified protein against the color intensity of each band. The serum concentration of each C3 in serum was then calculated from the standard curve.

Protein Sequencing. To obtain the NH_2 terminal sequences of the C3 chains, the intact molecules were reduced, subjected to electrophoresis, and electroblotted onto ProBlott membranes (Applied Biosystems) (15). The internal protein sequences of trout C3-3 and C3-4 were obtained by digesting them with the endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer Mannheim) (16). Digestion was conducted at a C3:enzyme ratio of 100:1 (wt/wt) in PBS, pH 7.5, with 0.1% SDS/0.1% trifluoroacetic acid. Separation of various peptides was performed using an Applied Biosystems 130A Micro Separation system on a reversed phase C4 column at 25°C with a flow rate of 200 μl/min. The C3 peptides were eluted with a 14-ml gradient of 0 to 63% acetonitrile containing 0.1% trifluoroacetic acid and detected at 214 nm. Fractions were analyzed by matrix-assisted laser desorption spectrometry (VG Tofspec; Fisons Pharmaceuticals Ltd, England), and fractions containing single peptides were subjected to gas-phase sequencing.

Determination of Trout C3-3 cDNA Sequence. A partial cDNA sequence of trout C3-3 was obtained by sequencing a 1.0-kb reverse transcription polymerase chain reaction (RT-PCR)-amplified C3-3 fragment. Total RNA was isolated from rainbow trout liver by the acid guanidine-thiocyanate-chloroform extraction method. cDNA was synthesized using an MMLV reverse transcriptase and random hexamer primer (GIBCO/BRL). PCR was performed using primers from the C3-3A clone (5′-AAGGGAATCTTCATAGTC-3′) and from the N-terminal sequence of the α-chain of C3-3: (5′-ACNACA(G)TCA(G)TTA(GT)ATNGT-3′). After initial denaturation for 5min at 95°C, amplification was performed in 28 cycles of 1 min at 95°C, 1 min at 48°C and 90 sec at 72°C, with a final elongation step of 10 min at 72°C.

The PCR fragment (1.0 kb) was subcloned into the pGEM-T vector using the pGEM-T vector system (Promega). DNA sequencing of both strands was performed according to Sanger *et al.* (17), using the Amersham Sequenase kit (United States Biochemical); each strand was sequenced at least two times.

Determination of the Ability of Trout C3 to be Fixed to the Surface of Various Complement Activators. The percentage of C3 deposited onto various complement activators upon complement activation was determined by incubating 20 μl of each activator (a 50% suspension) with 50 μl of trout serum or human serum (as a control) containing 30 μl (2 μCi) of ^{125}I-labeled purified trout C3 or Hu C3 protein in 10 mM PBS, pH 7.4, in the presence or absence of 2 mM Mg^{2+}EGTA or 10 mM EDTA. The reaction mixture was incubated at 20°C for trout serum and 37°C for human serum. Bound radioactivity was separated from free protein by centrifugation through 20% sucrose in 10 mM phosphate buffer, pH 7.4, containing 500 mM NaCl and 20 mM EDTA. The specific activity of the C3 proteins varied from 4 to 7.5 μCi/μg. The labeled C3 contained more than 90% native C3, as determined by their incubation with trout serum and rabbit erythrocyte ghosts in the presence or absence of EDTA (cleavage of C3 indicates the presence of iC3). For the sheep erythrocyte ghosts, the pellets obtained were solubilized by boiling in SDS sample buffer and analyzed by 9% SDS-PAGE under reducing conditions. The gel was then dried and subjected to autoradiography.

RESULTS AND DISCUSSION

During the process of cloning trout (*S. gairdneri*) C3-1 (14), a PCR product (the C3-3A clone) was obtained, whose deduced amino acid sequence was 50% identical to the corresponding area in trout C3-1 (Lambris, J. D., unpublished data). This finding led us to hypothesize that the trout might have more than one C3-related protein. The procedure used to search for such proteins was a modification of a purification method previously established for the isolation of C3 from a number of species (12). After several chromatographic steps two novel C3-related proteins (C3-3 and C3-4) were isolated. From 10 ml of trout serum, 4.5 mg of purified C3-1, 0.9 mg of C3-3, and 0.75 mg of C3-4 were obtained.

All three purified trout proteins were composed of an α- and β-chain (Fig. 1), with analogous chains having similar molecular masses. Thus, C3-3 and C3-1 had α- and β-chains of 112 kDa and 70 kDa, respectively; those of C3-4 were 107 kDa and 73 kDa, respectively. Both C3-3 and C3-4 contained Con A-binding carbohydrates in both chains, whereas C3-1 carried a Con A-binding moiety only in the β-chain (Fig. 1).

Primers derived from the C3-3A clone and from the N-terminal sequence of the α-chain of C3-3 were used to isolate a 1-kb PCR product by RT-PCR. The deduced amino acid sequence of this product matched both the partial protein sequence obtained from the N-terminus of the α-chain of C3-3 and those of the N-termini of several proteolytic fragments generated from C3-3. A search of protein databases indicated that the deduced amino acid sequence of the PCR product displayed the highest similarity to trout C3-1 (51.5% identity). The amino acid sequence of C3-4 was obtained by N-terminal microsequencing of endoproteinase Lys C generated-

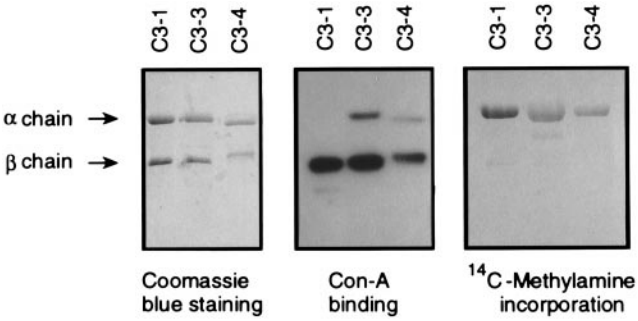

FIG. 1. Characterization of trout C3-1, C3-3 and C3-4. (*Left*) Purified proteins (3 μg) were resolved on 7.5% SDS-PAGE under reducing conditions and stained with Coomassie blue. (*Center*) SDS-PAGE identical to that in panel 1 was carried out, and proteins were electroblotted to a PVDF membrane, incubated with ^{125}I-labeled concanavalin A, washed, and subjected to autoradiography (12). (*Right*) Various forms of C3 (10 μg) treated with [^{14}C] methylamine (12) were electrophoresed, and the gel was treated with En^{3}hancer 228 (NEN) according to the manufacturer's instructions and subjected to autoradiography.

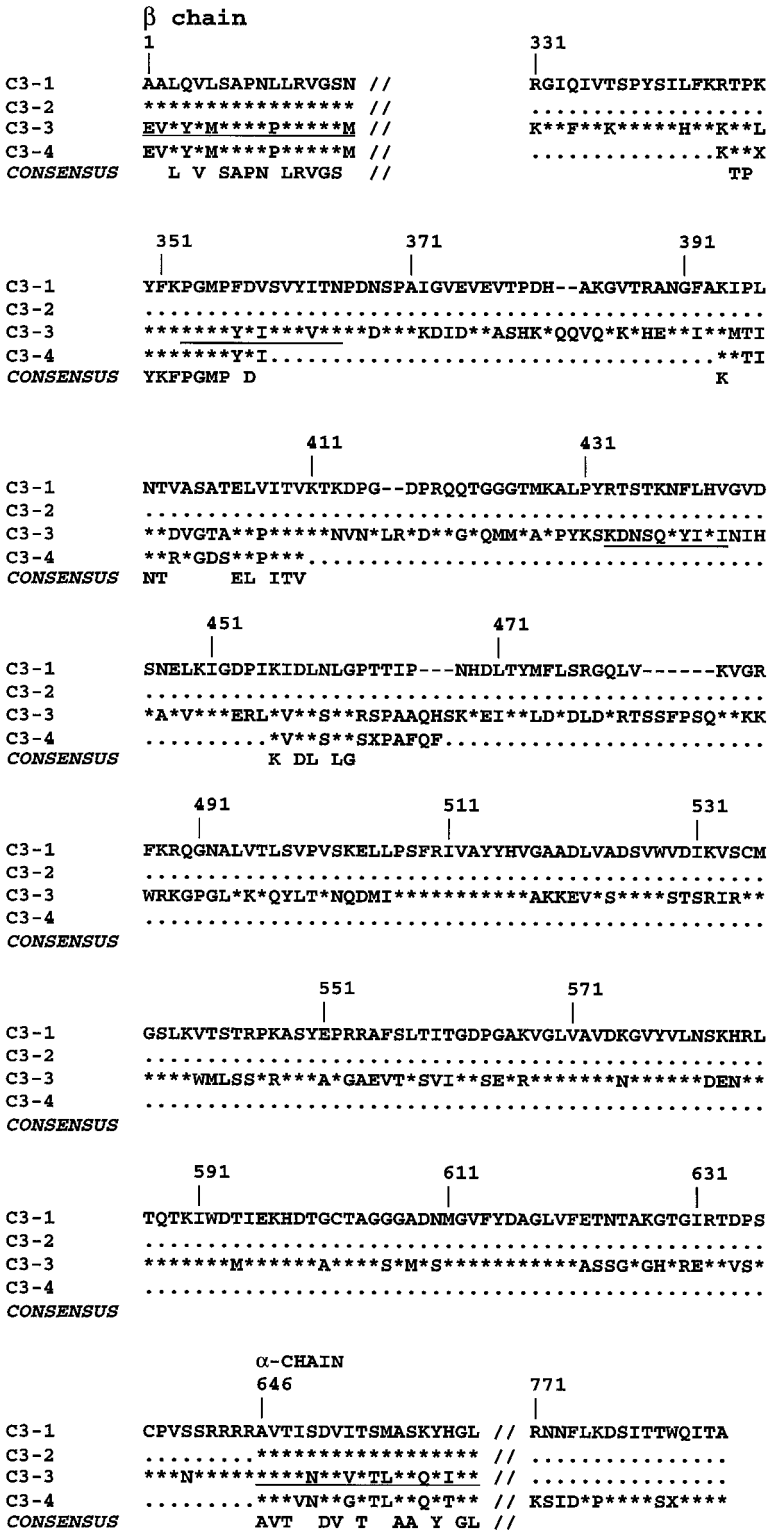

FIG. 2. Amino acid sequence comparison of trout C3-1, C3-2, C3-3, and C3-4. The amino acid sequence of trout C3-1 (14) and partial sequence of C3-3 were deduced from the nucleotide sequence of the cDNA clones isolated from a trout liver cDNA library. The N-terminal sequences of C3-2 subunits were determined previously (13). The amino acid sequence of C3-3 (underlined C3-3 residues) and C3-4 were obtained by N-terminal sequencing of their subunits and of peptides generated using the endoproteinase Lys C. Amino acid residues were numbered according to the sequence of C3-1 (14). Asterisks denote residues identical to those of C3-1; dots indicate the region for which the amino acid sequence was not determined; dashes indicate gaps introduced for maximum sequence alignment.

fragments of its α- and β-chains (16). Of 93 C3-4 residues determined by amino acid sequencing, 78 corresponded to the sequenced segment of C3-3. The C3-4 sequence was very similar to that of C3-3, with 68 identical residues (87%) and 3 (4%) conserved changes (Fig. 2). C3-4 was 60% identical to C3-1, with 56 identical residues and 12 (13%) conserved changes. The isolated C3-3 and C3-4 proteins were clearly different from the previously reported C3-2 protein (13), because the N-terminal sequences of the α-chains of C3-3 and C3-4 differed in 5 and 6 residues, respectively, from that of

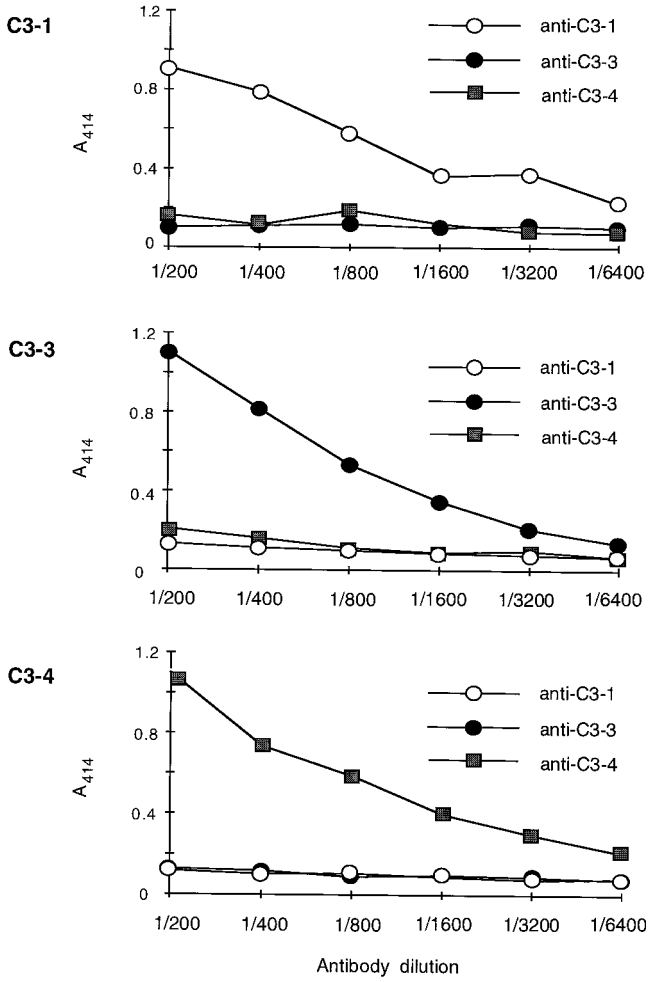

FIG. 3. Antibody reactivity with trout C3-1, C3-3, and C3-4. Reactivity of each form of C3 with rabbit antisera raised against the subunits of the purified proteins was detected by ELISA, as described in *Materials and Methods*.

C3-2. In addition, the molecular masses of the chains reported for C3-2 differ from those observed here for C3-3 and C3-4. All our attempts to isolate the C3-2 molecule from the trout sera analyzed in this study were unsuccessful. When the sequences of all three proteins were compared with those of C3 and various C3-related proteins (C4, C5, and α_2-macroglobulin) the highest similarity was found with the C3 homologs and the lowest (<23%) with α_2-macroglobulins.

To analyze the antigenic similarities of the various C3 proteins, to determine whether all three proteins are expressed in a single animal and to assess their relative serum concentrations, monospecific antibodies were generated by immunizing rabbits with isolated chains of C3-1, C3-3, or C3-4 that had been purified by SDS-PAGE (14). In ELISA experiments, each of these antibodies reacted specifically with the antigen against which it was raised but did not crossreact with the other C3 proteins (Fig. 3). In addition, an antibody to cobra venom factor reacted strongly with C3-1 but failed to recognize C3-3 and C3-4 (data not shown). All three proteins were expressed in a single animal, as assessed by immunoblotting of sera from 12 fish (six males and six females) (data not shown). The range of concentrations of trout C3-1, C3-3, and C3-4 in serum were 1.5 to 2 mg/ml, 0.3 to 0.4 mg/ml and 0.3 to 0.4 mg/ml, respectively.

One of the distinguishing characteristics of C3 is its ability to bind covalently to acceptor molecules on cell surfaces, complex carbohydrates or immune complexes (18, 19). This function has been attributed to the thioester bond localized in the α-chain, a moiety also present in two other plasma proteins, complement component C4 (20) and α_2-macroglobulin (21). The deposition of C3b on surface structures is important for the initiation of the membrane attack complex, for phagocytosis of foreign particles, and for the enhancement of effector cell-target cell contact. The presence of a thioester bond in C3-1, C3-3, and C3-4 was determined by analyzing the ability of each purified C3 protein to incorporate covalently [^{14}C] methylamine and to bind covalently to sheep erythrocyte ghosts. This analysis revealed the presence of a thioester bond in the α-chain of each of the three proteins (Fig. 1). When sheep erythrocyte ghosts that had been preincubated with radiolabeled C3 and trout serum were analyzed by electrophoresis, high molecular weight complexes migrating slower than the α-chain were observed. All three C3 proteins showed a similar banding pattern between 130–400 kDa (Fig. 4). These data indicate that despite the amino acid substitutions present in C3-3 and C3-4, the thioester site is functional. The presence of a 43-kDa band in the absence of EDTA in all three forms of C3 (Fig. 4) suggests that they are cleaved to iC3b (14), presumably by factors I and H.

Since all three C3 proteins contained a thioester bond, their capacity to participate in complement activation processes was investigated by assessing their ability to be fixed to zymosan particles (a potent activator of the complement system) (22). Unlike C3-1, C3-3, and C3-4 failed to bind to zymosan particles, since no C3 fragments were detected by their respective antisera after elution with 2-mercaptoethanol (16) (data not shown). Furthermore, when the three C3 proteins were radiolabeled, only C3-1 bound to zymosan particles after complement activation (Fig. 5).

When other surfaces were tested as complement activators, all three proteins were bound, but to different degrees. Trout C3-1 was similar in behavior to human C3, although its binding to sheep erythrocyte ghosts was lower than that of human C3. Furthermore, C3-1 did not bind to these ghosts through the alternative pathway. Sheep erythrocyte ghosts, in contrast to intact erythrocytes, bound human C3 (data not shown) in the presence of Mg^{2+}EGTA, and this binding might be mediated via proteins of the inner membrane. In the case of human C3 and trout C3 an increased binding to sheep erythrocyte ghosts in the absence of EGTA or EDTA (Fig. 5) was observed. Presumably this binding was mediated via the classical pathway and was initiated by the natural antibodies to sheep erythrocytes that are present in both human and fish serum. Both C3-3 and C3-4 bound to rabbit and sheep erythrocyte ghosts to a similar degree; in contrast, the binding of C3-1 to sheep erythrocyte ghosts under the same conditions was much lower than that to rabbit erythrocyte ghosts. Moreover, in the presence of Mg^{2+}EGTA, both C3-3 and C3-4 bound significantly, though to a low degree, to sheep erythrocyte ghosts; C3-1 did not bind. Taken together, these results suggest that the surface binding specificity of C3-3 and C3-4 differs from that of C3-1; in these experiments no differences in binding specificity were observed between C3-3 and C3-4. Examination of the classical pathway showed that all three proteins were able to bind to sensitized surfaces (data not shown).

These data demonstrate, for the first time, the existence of three different C3 molecules in one animal species. In this study, strong evidence was obtained to indicate that the isolated molecules are indeed C3: (*i*) they all have a chain structure similar to that of C3 from other species; (*ii*) they each contain a thioester bond in the α chain; (*iii*) their sequences show high similarity to the sequences of C3 from other species; (*iv*) they can be covalently bound to surfaces during activation of the classical and alternative pathways; (*v*) their cleavage patterns in serum during complement activation are similar to those of C3 proteins from other species; (*vi*) they can form alternative pathway C3 convertase with trout factor B in the presence of factor D (J.O.S. and J.D.L., unpublished work). It

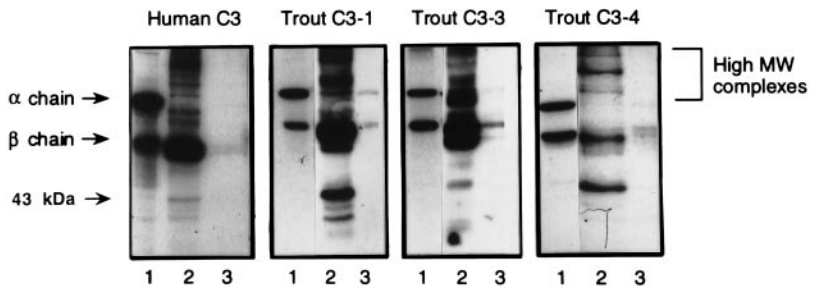

FIG. 4. Covalent binding of ^{125}I-labeled human C3 and trout C3-1, C3-3 and C3-4 to sheep erythrocyte ghosts. Sheep erythrocyte ghosts were incubated with ^{125}I-labeled human C3 or trout C3-1, C3-3, or C3-4 in the serum of the respective species, as described in *Materials and Methods*. Samples were incubated with (lane 3) or without (lane 2) 20 mM EDTA. ^{125}I-labeled C3 bound to erythrocyte membranes was separated from free iodinated protein by centrifugation through 20% sucrose. The pellets obtained were solubilized by boiling in SDS sample buffer and analyzed by 9% SDS-PAGE under reducing conditions. The gel was then dried and subjected to autoradiography. Lane 1 shows the radiolabeled C3.

is important to note that the specific amino acid sequences of the C3 variants (C3-3 and C3-4) caused them to differ from C3-1 in their nature of specific binding to particular surfaces. It is still unknown whether these differences result from a difference in affinity between C3-3 and C3-4 and the regulatory proteins of the complement system or from a change in the surface specificity of C3-3 and C3-4. Isolation of trout regulatory proteins should enable us to address these questions.

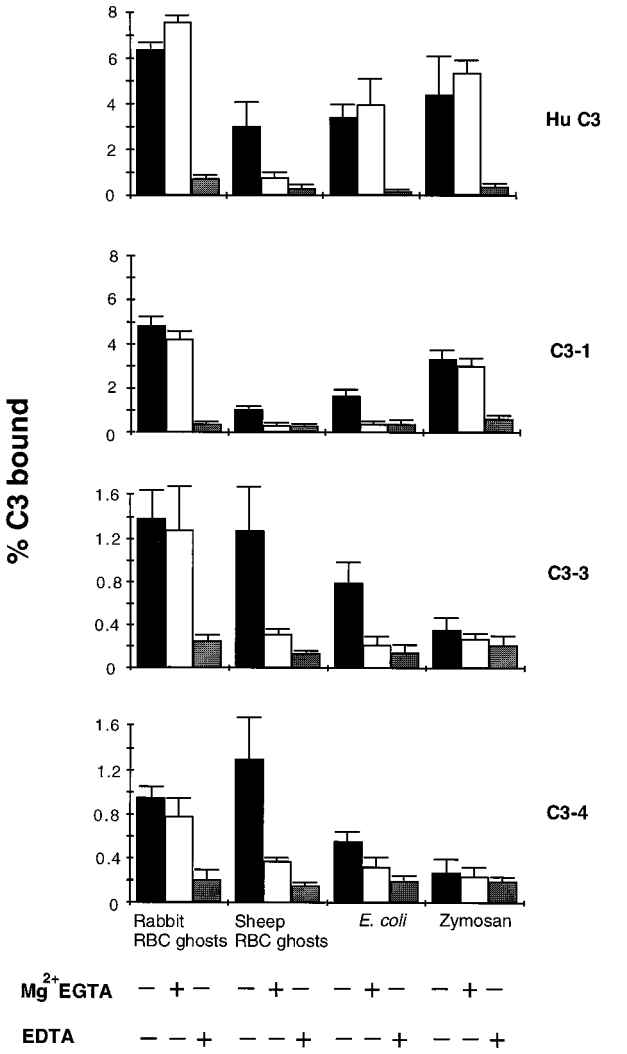

FIG. 5. Attachment of human C3 (Hu C3) and trout C3-1, C3-3, and C3-4 to various complement-activating surfaces. Each bar represents the mean ± SD of three different experiments (for details see *Materials and Methods*).

The coexistence of three forms of C3 in the same animal and the distinct nature of their binding specificities might serve to increase the number of possible pathogens recognized by these proteins and therefore enhance the protective effect of the organism's complement. A similar phenomenon occurs in humans: C4 exists in two forms, C4A and C4B. These two molecules have very few amino acid differences (13 substitutions in 1722 residues), but they have distinct binding specificities. Human C4A binds preferentially to surfaces carrying amino groups, whereas C4B has a higher affinity for those containing hydroxyl groups (20). The two proteins are the products of distinct genes that are organized in tandem.

Trout belong to the Salmonidae family. These fish are tetraploid, although some members of the family (such as *S. gairdneri*, the rainbow trout), are in the process of diploidization (7, 8). Our results suggest that during the tetraploidization event, the C3 locus was duplicated and one locus probably diverged, giving rise to the ancestor of C3-3 and C3-4; this ancestor was then duplicated a second time to generate the two proteins. It is unlikely that C3-3 and C3-4 are alternatively spliced products of a single gene, since the amino acid differences are scattered throughout different segments of both proteins. Although our data indicate that C3-3 and C3-4 are the products of different genes, a definitive conclusion can be drawn only after analyzing the genomic location of these gene(s). Since the genes encoding mammalian C3, C4, and C5 are not arranged in tandem but are located on different chromosomes, it is tempting to speculate that C4 and C5 are also the result of a similar genome duplication event, in which genes were duplicated from C3 or a common ancestor and evolved to C4 and C5. If this hypothesis is correct, then the duplication must have happened before the emergence of the salmonids, because trout have a functional classical pathway that includes a C5 molecule (23). In this case C3-3 or C3-4 would not be the ancestors of trout C4 and C5. The question to be addressed is whether the evolutionary emergence of this new class of C3 in trout led to the generation of a new C3-related protein family and whether similar proteins also exist in higher vertebrates and constitute such a C3-related protein family.

Tetraploidization and gene duplication events in other species have very often led to the generation of inactive products or products with completely different functions (24). It is of particular interest that despite their significant differences in amino acid sequence, the products encoded by the three C3 genes in the trout, are all functionally active.

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