Biosynthesis and Processing of a Human Precursor Complement Protein, pro-C3, in a Hepatoma-Derived Cell Line

Abstract. *A 180,000-dalton single-chain molecule (human pro-C3) is the precursor of the third component of human complement (C3), a disulfide-linked two-chain protein. The pro-C3 is converted by limited proteolysis to C3. The relationship between pro-C3 and C3 was established with the use of Hep G2, a cell line derived from a human hepatocellular carcinoma, which synthesizes at least 17 plasma proteins.*

The availability of well-characterized human cell lines of liver origin (*1, 2*) provides an important tool for examining the synthesis and processing of plasma proteins. We have used one of these cell lines (Hep G2) to elucidate the synthesis and posttranslational modification of the third component of human complement (C3); C3 is the most abundant of a series of plasma proteins that constitute the principal humoral effector of the immune system (*3*). Native human C3 has a molecular size of approximately 187,000 daltons and is composed of two disulfide-linked polypeptide chains of 115,000 and 75,000 daltons (*4*). The sites of C3 synthesis are the liver (*4–10*), monocytes and macrophages (*11–13*), and possibly fibroblasts (*14*).

Although single-chain putative precursors of complement proteins have been identified for components C3 (*9, 10, 14*), C4 (*15–22*), and C5 (*23, 24*), only pro-C4 and C4 have been definitely shown to be precursor and product (*19–22*). The existence of a precursor for human C3 has not been established. Patel and Minta (*10*) isolated a 197,000-dalton nondissociable protein from human serum by immunoadsorption with antiserum to C3, but this protein was not identified as an authentic C3 precursor. In fact, high molecular weight, covalently linked oligomers of C3 resulting from C3 cleavage, have been described (*25*); on the basis of size alone, these degradation products cannot be distinguished from an authentic precursor protein. We analyzed culture mediums and cell lysates of Hep G2 to determine whether human C3 is derived from a procomplement precursor and to elucidate its postsynthetic processing. We conclude that human C3 is synthesized as a single-chain precursor that gives rise, by limited proteolysis, to native functional C3.

Confluent cultures of Hep G2 cells (approximately 2×10^7 cells per T-75 flask) were incubated with L-[^{35}S]methionine; the cultures were then incubated in medium containing unlabeled L-methionine. At timed intervals, labeled C3 was immunoprecipitated from mediums and cell lysates (see legend to Fig. 1) and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*26*); and functional activity was measured with a hemolytic assay (*7*). The results indicate that C3 is synthesized as a single polypetide chain of approximately 180,000 daltons (Fig. 1A). Conversion of pro-C3 to native C3 was indicated by the gradual disappearance of pro-C3 (180,000 daltons) from the cellular material (lanes 1 to 5 in Fig. 1A), and the concomitant appearance of extracellular disulfide-linked two-chain (115,000 and 75,000 daltons) native C3 (lanes 6 to 9 in Fig. 1A). The medium from the last time point contained functionally active C3 (4.6×10^6 effective molecules per culture at 4 hours).

Since plasmin (plasminogen and urokinase) cleaves pro-C4 to C4 (*22*), we also

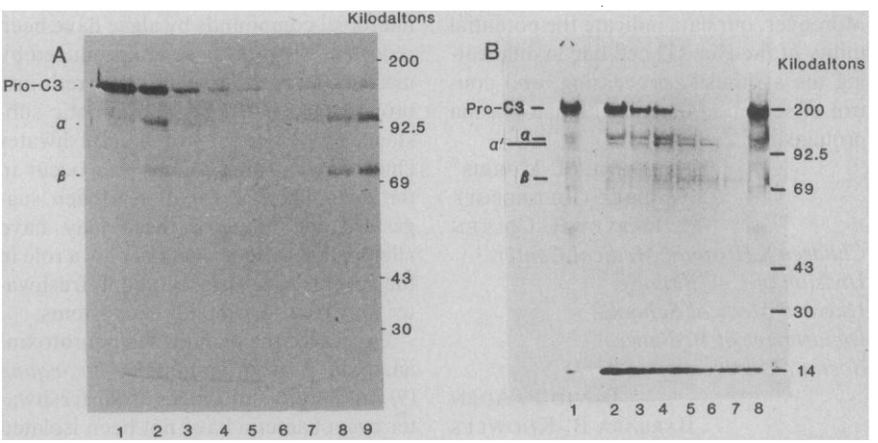

Fig. 1. (A) Synthesis of pro-C3 and secretion of C3 by Hep G2, a human hepatocellular carcinoma cell line. Five replicate cultures were each labeled with 250 μCi/ml of L-[^{35}S]methionine (1100 Ci/mmole; 250 μCi/ml) in 4 ml of Earle's minimal essential medium (MEM) minus methionine. After 30 minutes the medium was removed and the monolayers were washed with MEM. Fresh MEM was added to all but one culture, which was trypsinized; the cells were washed twice in 15 ml of MEM and frozen at −70°C. At 30, 60, 120, and 240 minutes, the medium was removed and the cells were collected and frozen as described above. Cells were thawed and immediately lysed in 4 ml of lysis buffer [0.5 percent (weight to volume) Triton X-100, 0.5 percent (weight to volume) deoxycholate, 50 m*M* tris, 100 m*M* KCl, 10 m*M* EDTA, and 2 m*M* phenylmethylsulfonyl fluoride (PMSF)], then frozen and thawed twice; the supernatants were transferred to a new tube after centrifugation at 10,000*g* for 30 minutes. Immunoprecipitation was carried out by incubating each sample (100 μl) with goat antiserum to human C3 (at a volume yielding 30 μg of precipitate at equivalence) and human C3 (at half the amount determined for equivalence, that is, a twofold antibody excess) for 1.5 hours at room temperature. Controls consisted of immunoprecipitates formed between rabbit antiserum to ovalbumin and ovalbumin in the presence of labeled medium or cell lysate. The immunoprecipitates were washed three times in the lysis buffer, once in 150 m*M* NaCl and once in acetone, then dried. Each was dissolved in sample buffer, reduced by boiling for 2 minutes with 50 m*M* dithiothreitol, and subjected to electrophoresis on 1.0 percent sodium dodecyl sulfate–9.0 percent polyacrylamide gel (*27*). The gel was stained and destained, soaked in En^{3}Hance (New England Nuclear) and water, dried, and exposed to film (Kodak X-Omat AR). Lanes 1 to 5 of the autoradiogram were immunoprecipitated from cellular lysates at 0, 30, 60, 120, and 240 minutes after the labeling period, and lanes 6 to 9 were from extracellular medium at 30, 60, 120, and 240 minutes after the labeling period. No detectable labeled protein was observed on autoradiographs of the control immunoprecipitates. (B) Conversion of pro-C3 to C3 by plasmin. Cellular lysate (250 μl) after a 30-minute period of labeling with L-[^{35}S]-methionine was incubated at 37°C with plasmin [1.25 μl of plasminogen and urokinase (with an absorbance at 412 nm of 0.033 min $^{-1}$) with 150 μl of Chromogenic Substrate S-2251 (Ortho Diagnostics, Raritan, New Jersey) and 750 μl of phosphate-buffered saline, *p*H 7.0]. At 2, 4, 8, 16, and 32 minutes after plasmin was added, 30 μl of lysate was removed to a tube containing 120 μl of 0.5 percent (weight to volume) Triton X-100, 0.5 percent (weight to volume) deoxycholate, 50 m*M* tris, 100 m*M* KCl, 10 m*M* EDTA, 2 m*M* PMSF, and benzamidine (final concentration, 20 m*M*). Two additional portions, incubated for 32 minutes at 37°C either with urokinase, equivalent to the amount present in the plasmin sample, or with buffer alone, were treated identically. All tubes were centrifuged; 120 μl of lysate was transferred to new tubes, and each was immunoprecipitated with carrier C3 and antiserum C3, washed, and subjected to electrophoresis as described in (A). Timed samples (2, 4, 8, 16, and 32 minutes) with plasmin are present in lanes 2 to 7. The lysate treated for 32 minutes at 37°C with urokinase alone is in lane 1 and the lysate incubated without plasmin for 32 minutes at 37°C is in lane 8.

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treated cellular lysates at 37°C with plasmin for measured time periods and then used immunoprecipitation and SDS-PAGE analysis. The 180,000-dalton pro-C3 was cleaved to peptides with mobilities identical to those of the α and β chains (115,000 and 75,000 daltons) of the C3 secreted by the hepatoma as well as to those of purified C3 protein from human plasma. Continued incubation resulted in further cleavage of the α chain to an *a'* chain (100,000 daltons) (Fig. 1B). Neither urokinase alone, in an amount present in the plasmin preparation (lane 1 in Fig. 1B), nor incubation of the lysate alone at 37°C (lane 8 in Fig. 1B) resulted in cleavage of pro-C3.

Previous studies had established, on the basis of the identity of partial amino acid sequences, kinetics of synthesis in cell culture, cell-free synthesis, and conversion in vitro of procomplement to native protein, that pro-C4 is the precursor of C4. We have now demonstrated that the third component of human complement is synthesized as a procomplement protein (pro-C3), and we have defined the precursor-product relationship between the single-chain pro-C3 and disulfide-linked two-chain native C3. Hence, in addition to their similar structural (*27*) and functional features, there are similarities in the biosynthesis and postsynthetic processing of C3 and C4. Moreover, our data indicate the potential utility of the Hep G2 cell line in delineating the synthesis, processing, and control of secretion of other human plasma proteins.

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Isolation of Chlorine-Containing Antibiotic from the Freshwater Cyanobacterium *Scytonema hofmanni*

Abstract. Scytonema hofmanni, *a filamentous freshwater cyanobacterium (blue-green alga), produces secondary metabolites which inhibit the growth of other cyanobacteria and green algae. A rapid, qualitative assay for this inhibition has been developed with* Synechococcus *as the test organism. This assay procedure has led to the isolation and characterization of an antibiotic (named cyanobacterin) from* Scytonema. *The antibiotic has a molecular weight of 430 and an empirical formula of* $C_{23}H_{23}O_6Cl$ *and contains a γ-lactone and a chlorinated aromatic nucleus. It inhibits the growth of various algae but has limited effect on nonphotosynthetic bacteria or protozoans and thus may have potential use as a specific algicide.*

The production and excretion of antimicrobial compounds by algae have been reported. Most of these are produced by marine flora, principally the red and brown algae (*1–3*). Some antibiotic substances have been reported in freshwater chlorophytes (*4*) and may also occur in the cyanobacteria (*5*). It has been suggested that some of these may have allelopathic effects and thus play a role in the species succession noted in freshwater (*6, 7*) and marine (*8*) ecosystems.

Except for the mammalian neurotoxin, anatoxin A from *Anabaena flos-aquae* (*9*), antibiotic substances from freshwater cyanobacteria have not been isolated or characterized. We now report the occurrence of an antibiotic in the freshwater cyanobacterium *Scytonema hofmanni* (UTEX 1581). We have isolated a pure compound from this alga and have partially characterized it with respect to chemical structure and biological activity.

The antimicrobial properties of *Scytonema hofmanni* were first observed in laboratory cultures while attempting to grow this organism with other species of algae (*10*). The effects were most obvious in petri plates where filaments of *S. hofmanni* and another alga were initially streaked at right angles to each other on the agar. Within 7 to 10 days, *S. hofmanni*, which grows rather slowly under our conditions, had cleared the area in its vicinity of almost any other species with which it was paired. To eliminate the possibility that this clearing was due to competition for nutrients, cell-free extracts of *S. hofmanni* were prepared and tested against *Synechococcus* sp. (ATCC 27146), a unicellular cyanobacterium, growing in liquid culture. Growth of *Synechococcus* was monitored by measuring the optical density of the cultures over a period of 2 weeks. Growth of *Synechococcus* was inhibited by crude extract protein (as little as 30 μg/ml), an indication of the presence of potent antibiotic activity (*11*). Similar results were obtained with medium from a *S. hofmanni* culture as the antibiotic source. After 2 weeks of growth, the *S. hofmanni* filaments were removed by filtration. The spent medium was then lyophilized to dryness, and the residue was resuspended in a small volume of distilled water. After filter-sterilization, the medium was added to *Synechococcus* cultures and it inhibited the growth of these organisms as did the cell extracts. Thus under the conditions of laboratory culture, it is probable that *S. hofmanni* actively excretes the antibiotic.

Scytonema hofmanni filaments or cell-

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