BIOSYNTHESIS OF PRO-C3, A PRECURSOR OF THE THIRD COMPONENT OF COMPLEMENT*

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The complement system is composed of 11 plasma proteins that, together with proteins of the properdin system, constitute an important humoral effector of immunological reactions (1). Several native serum complement (C) proteins have been shown to consist of two or more covalently bound polypeptide subunits. These include C1q (2), C3 (3), C4 (4), C5 (3), and C8 (1). Recently, a precursor form of the fourth component of guinea pig C was identified in studies of the cell-free synthesis of C4 and in homogenates of liver (5). The precursor of C4 (pro-C4) is a single polypeptide chain of mol wt 200,000, which is converted into native serum C4, a three chain protein of similar molecular weight (4, 6).

The possibility was considered that other multichain C proteins were also synthesized as single chain precursors in analogy to pro-C4 and to other known precursor proteins such as proinsulin (7). In order to test this hypothesis, we analyzed the size and subunit composition of the third component of C (C3) synthesized in tissue culture and under cell-free conditions.

Materials and Methods

Phenylmethylsulfonylfluoride (PMSF), reduced glutathione, HEPES, GTP, creatine phosphokinase (CPK), phosphorylase A, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, Mo. ATP (dipotassium salt) and phosphocreatine (dipotassium salt) were from Calbiochem, La Jolla, Calif. Ribonuclease-free sucrose and ovalbumin (OVA) were from Schwartz/Mann Div., Becton, Dickinson and Co., Orangeburg, N. Y. Tritiated leucine, 45 Ci/mmol, and a 14C-labeled amino acid mixture consisting of equal amounts of 14C-leucine (325 mCi/mmol), 14C-valine (251 mCi/mmol), 14C-isoleucine (321 mCi/mmol), and 14C-lysine (282 mCi/mmol) were obtained from New England Nuclear, Boston, Mass. Medium 199 (M-199), Medium 199 Special (lacking valine, leucine, isoleucine, lysine, and glutamine), and glutamine were purchased from Microbiological Associates, Bethesda, Md.; β-galactosidase and carbonic anhydrase from Worthington Biochemical Corp., Freehold, N. J.; NCS solubilizer from Amersham/Searle Corp., Chicago, Ill.

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1 Abbreviations used in this paper: BSA, bovine serum albumin; CPK, creatine phosphokinase; M-199, Medium 199; OVA, ovalbumin; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, Tris(hydroxymethyl)aminomethane.
Antisera. The preparation of rabbit antiserum to guinea pig C3 (8) and of guinea pig antiserum to OVA (6) has been described. Rabbit antiserum to BSA was purchased from Microbiological Associates. The globulin fraction of each was separated from antiserum by 40% (NH₄)₂SO₄ precipitation at 4°C (30 min). The precipitate was dissolved in 100 mM KC1-50 mM Tris(hydroxymethyl)aminomethane (Tris) (pH 7.5) (Tris-KCl buffer), dialyzed against Tris-KCl buffer, and centrifuged (10,000 g) immediately before use.

Liver Tissue Culture. Short-term cultures of minced guinea pig liver were established by methods described in detail previously (6). Briefly, guinea pigs weighing approximately 200 g were killed and a portion of the liver immediately removed and placed in ice-cold M-199. The liver was minced and washed, then 400 mg were incubated for 15 min at 37°C in M-199 Special. The medium was removed and replaced with M-199 Special containing glutamine, penicillin, and streptomycin plus 1.0 ml (100 μCi) of ¹⁴C-labeled amino acids and the tissue fragments were incubated for 3-4 h at 37°C in a humidified 5% CO₂/95% air atmosphere. The medium was then separated from the liver tissue by centrifugation and used as a source of extracellular (secreted) ¹⁴C-labeled C3.

Homogenization of Liver Tissue. After separation of the medium from the short-term tissue culture, the minced liver was washed with M-199. Then 400 mg of liver were homogenized in 8 ml of cold 0.5% sodium deoxycholate, 0.5% Triton X-100, 100 mM KC1, and 50 mM Tris-KCl (pH 7.3), rinsed several times, finely minced, and then homogenized in two volumes of the same buffer. The homogenate was centrifuged (8 min at 20,000 g) and the supernate was used as a source of intracellular ¹⁴C-labeled C3.

Guinea Pig Liver S-20. The preparation of S-20, a source of polysomes and soluble factors required for protein synthesis, was described in detail previously (5). A guinea pig (approximately 400 g) was killed, a portion of the liver removed and immediately placed in ice-cold homogenization buffer (0.25 M sucrose, 50 mM KC1, 5 mM MgCl₂, 3 mM glutathione, and 50 mM HEPES, pH 7.3), rinsed several times, finely minced, and then homogenized in two volumes of the same buffer. The homogenate was centrifuged (8 min at 20,000 g) and the supernate was used as a source of intracellular ¹⁴C-labeled C3.

Cell-Free Synthesis of C3. A portion of the S-20 preparation (0.6 ml) was incubated for 90 min at 25°C in a mixture containing 5 mM MgCl₂, 100 mM KC1, 2 mM ATP, 0.5 mM GTP, 15 mM phosphocreatine, 10 μg/ml CPK, 19 amino acids (40 μM each) excluding leucine, 10 μM ³H-leucine (500 μCi/ml), 1.8 mM reduced glutathione, and 30 mM HEPES (pH 7.3), in a final vol of 1.0 ml in sterile Brinkman microfuge tubes (Curtin Matheson Scientific, Inc., Medford, Mass). After incubation, an equal volume of a solution containing 1% deoxycholate, 1% Triton X-100, 6.7 mM leucine, 100 mM KC1, and 50 mM Tris HCl (pH 7.5) was added to the incubation mixture followed by centrifugation at 100,000 g for 2 h at 2°C to sediment polysomes.

Assay of Total Protein Synthesis. Samples of 5 μl each were precipitated on Whatman 3 MM filter paper disks with TCA and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) following exactly a previously described procedure (5).

Immunoprecipitation. Purified guinea pig C3 (9) (330 μg/ml), BSA (1 mg/ml), or OVA (0.5 mg/ml) served as carriers for immunoprecipitation. Reaction mixtures were prepared containing 1.27 μl C3, 1.6 μl BSA (or 0 μl OVA), 190 μl of sample, and 95 μl 1% deoxycholate, 1% Triton X-100, 100 mM KC1, and 50 mM Tris-KCl (pH 7.5). The mixture was centrifuged (10,000 g) then in separate tubes C3 was precipitated with 12.7 μl anti-C3, the controls with 32 μl anti-BSA (or 20 μl anti-OVA). The amount of antibody was adjusted in each case to provide a twofold antibody excess and to yield a 30 μg immune precipitate after 1 h incubation at room temperature. After washing, the immune precipitates were either dissolved in NCS and counted in a liquid scintillation spectrometer, or they were dissolved and separated on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), as described below.

SDS-PAGE. The procedure according to Fairbanks et al. (10) for the preparation and electrophoresis of 5.6% polyacrylamide-1% SDS gels was followed. Immune precipitates were dissolved in SDS buffer, boiled, and electrophoresed as previously described (5). To assay radiolabeled protein, the gels were sliced into 2-mm segments, each placed in scintillation vials, and incubated with 1 ml of a NCS-toluene mixture (1:1) at 45°C for 18 h. Then 10 ml of scintillation fluid (0.5% 2,5-diphenyloxazole and 0.01% p-bis(2-(5-phenyloxazolyl))-benzene in toluene) was added, the vials
were cooled and stored for 24 h at 4°C, and radioactivity counted by scintillation spectrometry. Parallel gels with standard molecular weight markers were run and stained with Coomassie Blue. Markers were human spectrin (a gift from Dr. S. Lux, Children's Hospital Medical Center, Boston, Mass.), β-galactosidase, phosphorylase A, BSA, OVA, and carbonic anhydrase. Plotting of standard curves was done according to Weber and Osborn (11), using mol wt of 220,000 and 240,000 for spectrin.

Results

Medium harvested from short-term cultures contained hemolytically active C3 at a concentration of about $3.0 \times 10^6$ effective molecules/ml. Of the total protein secreted by the liver (TCA precipitable, 40,000 cpm/100 μl material) approximately 6% (2,300 cpm) was specifically precipitated by antiserum to C3. This amount was significantly greater than the proportion of C3 to total protein detected intracellularly or under cell-free conditions. That is, approximately 1% (500 cpm) of the total intracellular radiolabeled protein (54,000 cpm) and 0.2% (4,000 cpm) of total protein (1,800,000 cpm) synthesized and released from polyribosomes under cell-free conditions was precipitated by antiserum to C3. Extracellular $^{14}$C-labeled C3, secreted by liver fragments in culture, migrated as a single peak on SDS-PAGE in the absence of reducing agent (dithiothreitol) (Fig. 1 A). As estimated from standard markers, it had an apparent mol wt of 180,000 daltons (average of three experiments). Radiolabel in the control immunoprecipitates distributed uniformly throughout the gels. Under reducing conditions, C3 secreted by the liver fragments dissociated into two subunits of mol wt 120,000 and 76,000 (Fig. 1 B). Medium from starch-induced peritoneal macrophage cultures secreted hemolytically active C3 of size and subunit composition similar to that secreted by liver (data not shown).

Analysis on SDS-PAGE of intracellular C3, obtained from the liver homogenate, revealed a single peak of radioactivity under reducing conditions (Fig. 2 A). Its size was estimated to be about 170,000 daltons. Under cell-free conditions, newly synthesized C3 also had an approximate mol wt of 170,000 and its mobility was unchanged in the presence of dithiothreitol (Fig. 2 B). In addition to the major peak of 170,000 daltons, a small amount of radiolabeled material was present in the 70,000-40,000 dalton region.

In order to directly compare the size of intact C3 secreted by liver in culture with C3 synthesized under cell-free conditions, the cell-free product ($^3$H labeled) and culture medium ($^{14}$C labeled) were immunoprecipitated, the precipitates washed, and then mixed and subjected on the same gel to SDS-PAGE under nonreducing conditions. The results (Fig. 3) showed that they were of identical molecular size.

Discussion

Earlier reports indicated that C3 is synthesized in liver (12–14) and cells of the macrophage/monocyte series (15–17). In addition, it was shown that C3 secreted by human monocytes in culture has a molecular weight and subunit composition similar to that of native human serum C3; i.e. consists of two polypeptide chains.

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of mol wt 118,000 and 77,000. In the present report, the corresponding guinea pig protein secreted in both liver and peritoneal macrophage cultures was found to have a similar size and subunit structure. In contrast, intracellular C3 from liver homogenates and C3 synthesized under cell-free conditions consisted of a single polypeptide chain suggesting that C3 is synthesized and released from polysomes as a precursor (pro-C3). In this respect, the synthesis of both C3 and C4 (5) appear to be similar.

The conversion of proinsulin to insulin is the result of excision of a relatively large molecular weight peptide leading to a significant difference in size between the precursor and insulin itself (18). Similar observations have been made in the case of several other precursor proteins (19-22). With regard to pro-C4 or pro-C3 and their respective extracellular native products (C4 and C3), no significant loss of material apparently occurs in conversion of the precursor to the native protein. In fact, pro-C4 is slightly smaller than C4, possibly due to a
Fig. 2. SDS-PAGE of immunoprecipitates: (A) liver homogenate (intracellular material); anti-C3, anti-BSA in the presence of 50 mM dithiothreitol DTT; (B) products of cell-free protein synthesis, released from polysomes; anti-C3, anti-OVA in presence of 50 mM DTT.

Fig. 3. SDS-PAGE. Anti-C3 immunoprecipitates of cell-free products (3H-labeled) were combined with anti-C3 immunoprecipitate of liver tissue culture medium (14C-labeled) and electrophoresed in the absence of dithiothreitol in the same gel, then sliced and counted under double-label conditions.
difference in carbohydrate content. These data are most consistent with the hypothesis that conversion of pro-C3 or pro-C4 to the native extracellular proteins does not require excision of a large peptide, but the total sizes of these precursor proteins and the relative imprecision of the estimates of molecular weight preclude a definitive conclusion at the present time.

Conversion of pro-C3 to C3 is similar to conversion of Cls to its active form, Cls (23) or prothrombin to thrombin (24). For instance, evidence has been presented that activation of Cls (the zymogen) to Cls (the active esterase) results from limited proteolysis, converting a single chain molecule (Cls) to one with two chains linked by a disulfide bridge (Cls). Cls circulates in plasma in the C1 complex, but thus far no evidence for extracellular pro-C3 or pro-C4 (5, 6) has been obtained. Instead, C3 and C4 circulate as multichain proteins which require still further cleavage to generate biologically active proteins (1).

Evidence has been obtained that the light and heavy chains of gamma globulin are synthesized separately and then assembled to form the native molecule (25). The finding of single chain precursor proteins for C3 and C4 (5) raises the possibility that post-translational assembly of polypeptide subunits does not occur in production of other multichain C components. Studies of cell-free synthesis, as well as tissue culture methods, will make it possible to test the validity of this conclusion.

Summary

A precursor of the third component of complement, pro-C3, was detected in studies of cell-free synthesis and intracellularly in homogenates of liver tissue cultures. The molecular weight of pro-C3 was indistinguishable from that of intact native C3 secreted in vitro by liver or peritoneal macrophages, but its structure was different. Pro-C3 is a single polypeptide chain, whereas C3 secreted by cells in culture consists of two polypeptide chains (mol wt 120,000 and 76,000) linked by disulfide bonds.

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