Molecular Basis of Complement C3 Deficiency in Guinea Pigs

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Abstract

In experiments to ascertain the biochemical basis of a genetically determined deficiency of the third component of complement (C3) in guinea pigs, we found that C3-deficient liver and peritoneal macrophages contain C3 messenger RNA of normal size (~ 5 kb) and amounts, that this mRNA programs synthesis of pro-C3 in oocytes primed with liver RNA and in primary macrophage cultures. In each instance, heterodimeric native C3 protein was secreted with normal kinetics but the C3 protein product of the deficient cells failed to undergo autolytic cleavage and was unusually susceptible to proteolysis. These data and a selective failure of C3 in plasma of deficient animals to incorporate [^{14}C]methylamine suggested either a mutation in primary structure of the C3 protein or a selective defect in co- or postsynthetic processing affecting the thiolester bridge, a structure important for C3 function. A mutation in the primary structure of C3 was ruled out by comparison of direct sequence analysis of C3 cDNA generated from two C3 deficient and two C3 sufficient guinea pig liver libraries. Three base pair differences, none resulting in derived amino acid sequence differences were identified. Finally, restriction fragment length polymorphisms were identified in the C3 gene that are independent of the deficiency phenotype. This marker of the C3 gene permits testing of these hypotheses using molecular biological and classical genetic methods. (*J. Clin. Invest.* 1990. 86:96–106.) Key words: immunodeficiency • complement component C3

Introduction

The importance of the complement (C)[1] system in host defenses and immunopathology has been fully appreciated in the study of congenital and acquired complement deficiencies. The third component of complement (C3) in particular plays a pivotal role in many facets of the inflammatory response. Alper et al. (1, 2) first described homozygous deficiency of C3 in a child with a history of recurrent infections. The principal pathogens affecting this child, as in most C3-deficient individuals (3–6), were encapsulated bacteria, thus helping to establish in vivo the importance of C3 in opsonization, phagocytosis, and intracellular killing of these organisms. However, subsequent reports of C3-deficient individuals have presented conflicting data on defects in other C3-dependent functions such as immune adherence, development of leukocytosis in response to infection, vasopermeability, and antibody response (1–11). One possible explanation for these conflicting data is that, in C3 deficient individuals, C3 is synthesized and secreted, albeit at markedly reduced rates. Some C3 functions might therefore be retained, especially in extravascular sites. This suggestion is supported by observations that monocytes isolated from individuals with < 0.01% normal serum levels of C3, produced C3 at a rate ~ 25% of normal (12). Thus, although the hepatocyte is the primary source of plasma C3, studies of C3 biosynthesis in monocytes, fibroblasts and synovial tissue from patients with rheumatoid arthritis (13) suggested that regulation of C3 synthesis is independent in hepatocytes and other cells in extrahepatic sites. Ethical considerations and other technical constraints, however, did not permit further exploration of these questions in C3-deficient humans.

An isolated genetically determined deficiency of C3 in the guinea pig has recently been described (14). These animals were derived from an inbred strain 2 colony. Sera from C3 deficient (C3D) guinea pigs contain ~ 5% of normal C3 as assessed functionally and antigenically, i.e., the C3 appeared to have approximately normal specific hemolytic activity. Preliminary studies (14) suggested that peritoneal macrophages and hepatocytes from the C3D animals produced normal amounts of C3 and studies of the clearance of ^{125}I-radiolabeled normal guinea pig C3 in C3D guinea pigs demonstrated a normal catabolic rate. It is interesting that C3-deficient guinea pigs mounted only a minimal primary antibody response to $\phi \times 174$, a T cell-dependent antigen, and failed to undergo isotype switching from IgM to IgG with booster immunizations (15). This inadequate antibody response has also been found in deficiencies of complement components of the classical pathway (16, 17); all are believed to be due to deficiency of activated C3.

The availability of a small animal model deficient in C3 prompted further studies of the molecular basis for C3 deficiency. In this report we show that cells from C3D animals express C3 mRNA similar in size and amount to that found in normal cells. The C3D mRNA programs synthesis of a C3 protein that fails to undergo autolytic cleavage after heating in SDS, a property associated with the presence of a thiolester bridge in the α chain. In addition, a restriction length polymorphism of the C3 gene has been identified in C3D and some, but not all, C3-sufficient guinea pig strains, providing a marker for the C3 gene independent of the deficiency phenotype.

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1. *Abbreviations used in this paper:* C, complement; C3, third component of complement; C3D, C3 deficient; DOC, sodium deoxycholate; PCR, polymerase chain reaction.

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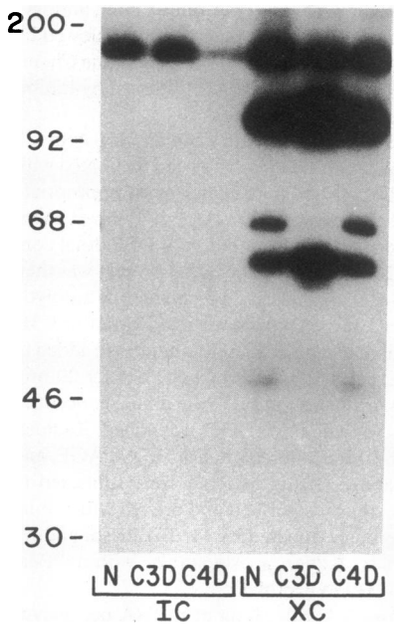


Figure 1. Biosynthesis of C3 by adherent peritoneal macrophages from normal (N), C3 deficient (C3D), and C4 deficient (C4D) guinea pigs. Autoradiograph of reduced SDS-PAGE analysis of intracellular (IC) pro-C3 and extracellular (XC) native C3 precipitated from culture media.

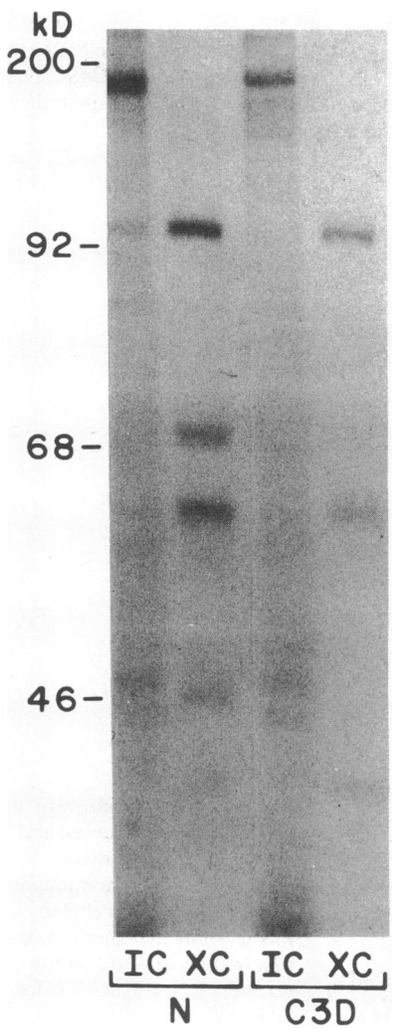


Figure 2. Biosynthesis of C3 in Xenopus oocytes primed with liver mRNA from normal (N) or C3D guinea pigs. Pro-C3 (intracellular) and C3 (extracellular) are analyzed by SDS-PAGE under reducing conditions.

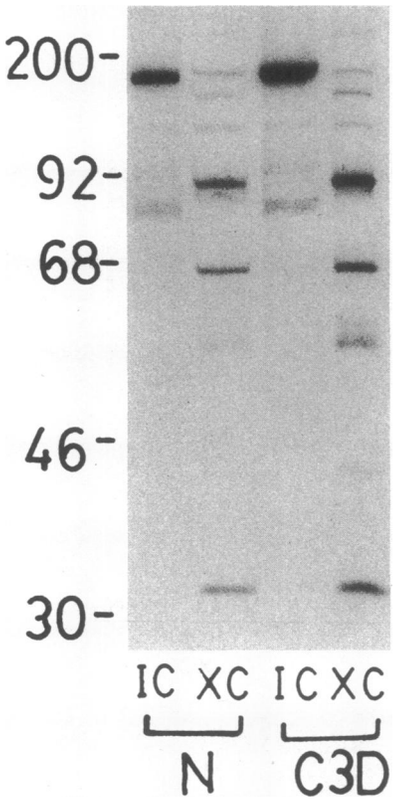


Figure 3. Biosynthesis of C4 in normal and C3D guinea pig peritoneal macrophages. Analysis of intracellular (IC) pro-C4 and extracellular (XC) native C4 on SDS-PAGE.

Methods

Cell culture. Peritoneal cells were harvested in HBSS without calcium and magnesium from normal Hartley, C3D, and C4D guinea pigs (C4D guinea pig plasma contains C3 at normal concentrations). The cells were isolated by centrifugation and resuspended in medium 199 (Gibco Laboratories, Grand Island, NY) with 50 U penicillin and 50 μg streptomycin (Gibco Laboratories) per ml supplemented with 10% heat inactivated (56°C for 2 h) fetal calf serum (Irvine Scientific Co., Santa Ana, CA) to 4×10^6/ml. 0.5 ml of the cell suspension was placed in 16-mm flat-bottom tissue culture wells (Corning Glass Works, Corning, NY) and the cells were allowed to adhere for 2 h. The monolayers were extensively washed with HBSS (viability was assessed by trypan blue exclusion, differential counts by Wright's stain and stained for esterase) and incubated for specified time periods at 37°C in Dulbecco's modified essential medium (DMEM; Gibco) lacking methionine and containing [^{35}S]methionine (~ 1,000 Ci/mmol; ICN Biomedicals, Irvine, CA). At timed intervals media were collected, cleared of cell debris by centrifugation at 600 *g* for 15 min, then stored at −70°C. Macrophage cultures were then washed extensively with HBSS and lysed by freeze-thawing in a solution of 50 mM Tris-HCl (pH = 7) containing 100 mM KCl, 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO) 0.5% sodium deoxycholate (DOC; Sigma), 2 mM PMSF (Sigma), and 10 mM EDTA.

Immunoprecipitation and analytical gel electrophoresis. Radiolabeled proteins were assayed as previously described (18). Briefly, aliquots of cell lysate or medium were incubated overnight at 4°C in 1% Triton X-100/1% SDS/0.5% DOC with excess antibody. A suspension of formalin fixed *Staphylococcus aureus* bearing protein A (IgSorb; The Enzyme Center, Boston, MA) was then added to each sample and incubated for 1 h at 4°C. After extensive washing, the antigen-antibody complex was released by boiling in sample buffer (0.05 Tris, pH = 6.8, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue), and applied to 9% SDS-PAGE under reducing conditions. After electrophoresis, gels were stained in Coomassie brilliant

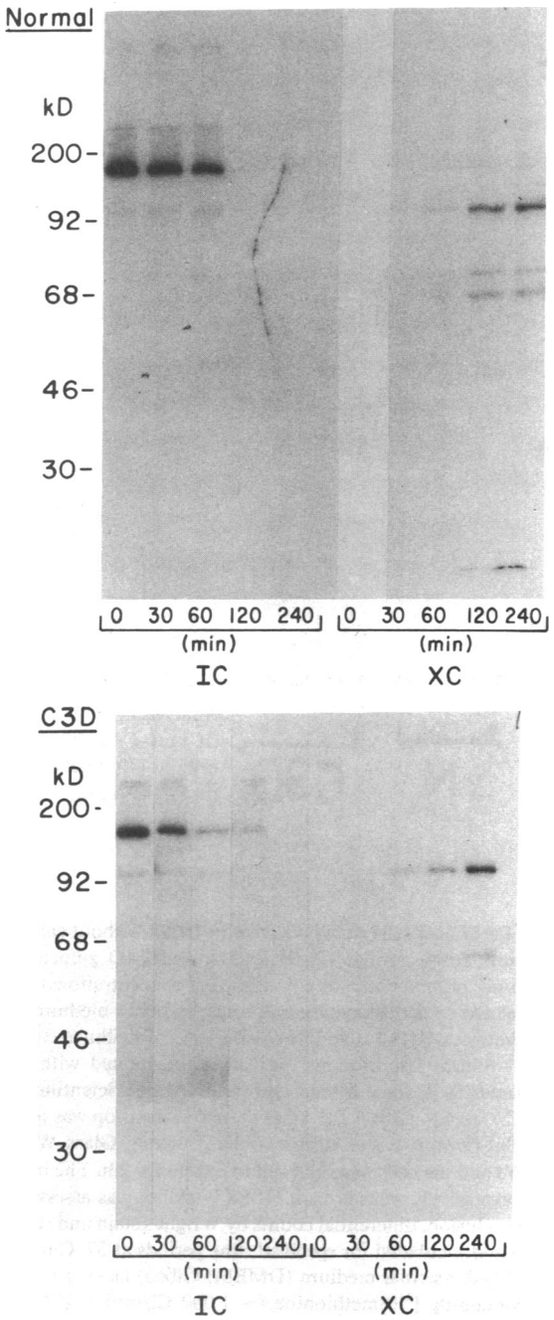


Figure 4. Kinetics of C3 secretion from normal and C3D macrophages. Cells were incubated with [^{35}S]methionine-containing media for 30 min, then washed and refed with fresh unlabeled media. C3 was immunoprecipitated from intracellular and extracellular samples at indicated time points.

blue, destained, impregnated with 2.5-diphenyloxazole (EN^{3}HANCE; New England Nuclear, Boston, MA), and dried for fluorography on XAR film (Eastman Kodak Co., Rochester, NY).

Antibodies. Antiserum to guinea pig C4 was raised in C4-deficient guinea pigs (19) by previously described methods (18). Antiserum to guinea pig C3 was raised in albino rabbits by subcutaneous injection of 1.0 ml of a mixture containing equal volumes of purified guinea pig C3, 1.0 mg/ml (supplied by M. Thomas, Washington University School of Medicine, St. Louis, MO) and complete Freund's adjuvant, followed by two injections at 2-wk intervals of 0.5 mg C3 in incomplete Freund's adjuvant. Blood was drawn 5 d after immunization, and IgG antibody was purified by ammonium sulfate precipitation followed by ion exchange column chromatography (DE52; Pharmacia Fine Chemicals, Piscataway, NJ). Antibody specificity was determined by inhibition of complement lysis and ouchterlony analysis.

C3 proteolysis. Media were harvested from normal Hartley or C3 deficient guinea pig macrophage cultures that had been incubated with [^{35}S]methionine for 4 h. Aliquots were incubated with appropriate concentrations of TPCK-treated trypsin (Sigma) for 4–5 min at room temperature. The reaction was stopped by addition of DFP (final concentration 1 M) at 37°C for 20 min. Radiolabeled C3 protein was then isolated and applied to SDS-PAGE as described above. For autolytic cleavage studies, 50-μl aliquots of media from normal Hartley or C3D macrophages previously incubated with [^{35}S]methionine were added to 750 μl of 0.1 M Tris acetate, pH = 8.5, and 0.1% SDS. After 20 min incubation at 75°C, 200 μl of a stock solution to yield final concentrations of 1% Triton X-100, 1.0% SDS, 0.5% DOC, was added. Radiolabeled C3 was then analyzed by immunoprecipitation, SDS-PAGE, and fluorography, as described above. 10 μg of RNA were subjected to Northern blot analysis as previously described and probed with ^{32}P-labeled mouse C3 cDNA generously donated by M. Takahashi and M. Nonaka (Kanazawa University, Kanazawa, Japan) or with a ^{32}P-labeled full length guinea pig C3 cDNA probe.

Xenopus oocytes were injected with 50 ng of mRNA per oocyte. The oocytes were incubated in microtiter wells (Corning Medical Instruments) containing 40 μl of Barth's medium/4 oocytes per well at 25°C for 6 h to allow puncture healing and recruitment of mRNA; the oocytes were then refed with DMEM containing 1 mCi/ml of [^{35}S]-methionine. After 50 h of incubation at 25°C, the media were removed. The oocytes were rinsed with PBS and homogenized with a loose fitting Dounce homogenizer (Fisher Scientific, Pittsburgh, PA) in 0.5 ml of 15 mM Tris, pH = 7.5, 50 mM NaCl, and 2 mM PMSF. The homogenates were centrifuged at 100,000 *g* for 5 min and aliquots then immunoprecipitated for C3, as described above.

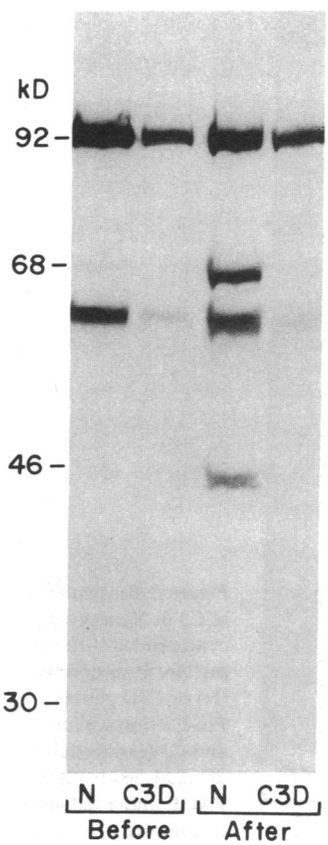


Figure 5. Autolytic cleavage of C3 synthesized in normal and C3D macrophage cultures. SDS-PAGE of C3 immunoprecipitated from extracellular media before and after incubation in 0.1% SDS, 0.1 M tris acetate, pH = 8.5, at 75°C for 20 min.

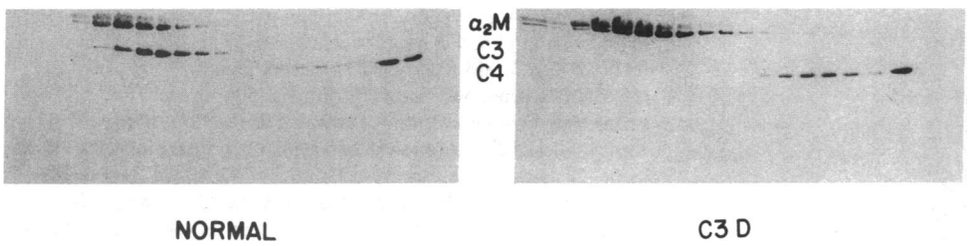


Figure 6. Incorporation of [^{14}C]-methylamine into guinea pig α_2 macroglobulin, and the α chain of C3 and C4. Normal and C3D guinea pig sera were fractionated on a Q Sepharose column. Fractions were incubated with [^{14}C]-methylamine and analyzed by SDS-PAGE and autoradiography. (*A*) Normal, (*B*) C3 deficient guinea pig serum. No [^{14}C]methylamine was incorporated into the C3 in the C3D serum fractions (B).

DNA analysis. 1 g liver fragments from C3D, C4D, C2D, and normal Hartley guinea pigs were digested in 0.5% SDS, 0.5 M EDTA, pH = 8, and 100 μg/ml proteinase K for at least 6 h at 50°C. DNA was extracted from digested solutions by phenol/chloroform, dialyzed against 1,000 times the volume of 10 mM Tris, pH 8, 1 mM EDTA, and stored at 4°C. Restriction endonuclease digestion of 10-μg aliquots was performed for more than 18 h, and the samples were subjected to agarose gel electrophoresis, transferred to nitrocellulose paper, and hybridized with a ^{32}P-labeled full length guinea pig C3 cDNA probe.

Methylamine incorporation in serum C3. A 4 cm × 1 cm Q Sepharose Fast Flow (Pharmacia Fine Chemicals) column was equilibrated with 10 mM Tris, 5 mM EDTA, 50 mM ϵ-aminocaproic acid, 0.2% Na azide, 0.2 mM PMSF, 100 mM NaCl, pH 7.4. 2 ml of normal or C3D guinea pig serum was applied and eluted with a 20-ml gradient to 500 mM NaCl in the same buffer. 1-ml fractions were collected. For methylamine incorporation, 22.5-μl aliquots of each fraction were incubated with 2.5 μl of 2 M Tris, pH 8.8, containing 50 mM [^{14}C]-methylamine, 48 mCi/mmol (New England Nuclear) for 30 min at 37°C, then were reduced and subjected to gel electrophoresis on a modified Laemmli gel (20). The gel was stained with Coomassie blue, dried, and exposed to x-ray film at −70°C.

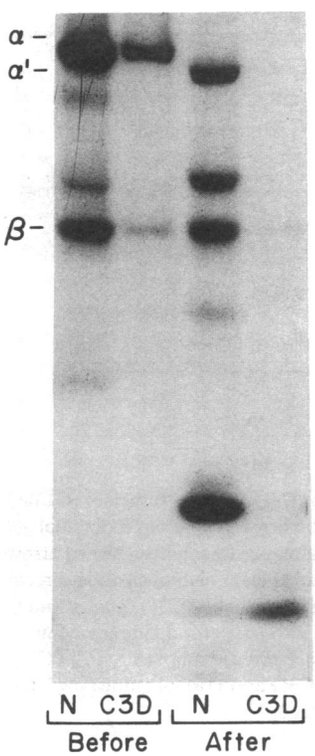


Figure 7. Trypsin cleavage of C3 from normal (N) and C3D guinea pig peritoneal macrophages. Native C3 immunoprecipitated before and after incubation with trypsin. Trypsin cleavage generates an α' fragment from normal C3 but no immunoprecipitable C3 protein in the C3D sample.

RNA. Total liver RNA was isolated from three C3-sufficient (Hartley, one animal, C4-deficient subline of NIH multipurpose guinea pigs, two animals) and two C3-deficient guinea pig livers according to previously described techniques (21). The polyadenylated RNA fraction was isolated by affinity chromatography on oligo-dT cellulose (22).

Construction of cDNA libraries. The cDNA synthesis system was purchased from Bethesda Research Laboratories (Bethesda, MD); restriction enzymes, T_4 DNA polymerase, and T_4 DNA ligase were purchased from New England Biolabs (Boston, MA); dNTPs were purchased from Pharmacia; *S*-adenosyl methionine and Eco RI methylase were purchased from Promega Co. (Madison, WI). Radionuclides [^{32}P]α-dCTP (800 Ci/mmol) and [^{35}S]α-dATP were purchased from New England Nuclear; oligo-dT cellulose type 3 was purchased from Collaborative Research (Bedford, MA); nitrocellulose HAW filters were purchased from Millipore Corp. (Bedford, MA); phosphorylated Eco RI linkers, lambda ZAP, XL-1 Blue, and Gigapak were purchased from Stratagene (La Jolla, CA); sequenase DNA sequencing kit was purchased from U. S. Biochemicals (Cleveland, OH); SP6/T7 Transcription kit was purchased from Boehringer-Mannheim (Mannheim, West Germany).

The C4- and C3-deficient poly A$^+$ was used to direct cDNA synthesis, which was then cloned into lambda ZAP by a modification of the methods described by Okayama and Berg (23, 24). Oligo-dT (12–18) or specific oligonucleotides designed from the guinea pig C3 sequence were utilized as primers for first strand synthesis in generating the primer extension libraries from one additional C3-sufficient (C4 deficient) and C3-deficient animal.

Isolation of C3 cDNA clones. cDNA libraries were plated at a density of ~ 8,000 plaque-forming units per 100-mm dish. Developed plaques were transferred to nitrocellulose filters. Filters were denatured, neutralized, baked in vacuo at 80°C for 2 h, prehybridized for 2 h at 42°C in 6× SSC, 1× Denhardt's solution, 0.5% SDS, 1% dextran

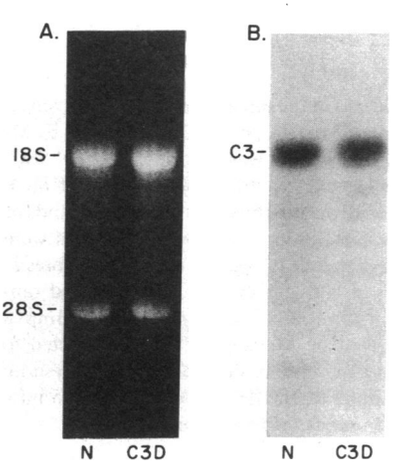


Figure 8. Northern blot analysis of equivalent amounts of RNA isolated from normal and C3D liver visualized under ethidium stain (*A*) and autoradiography (*B*). The blot was probed with a full length C3 cDNA.

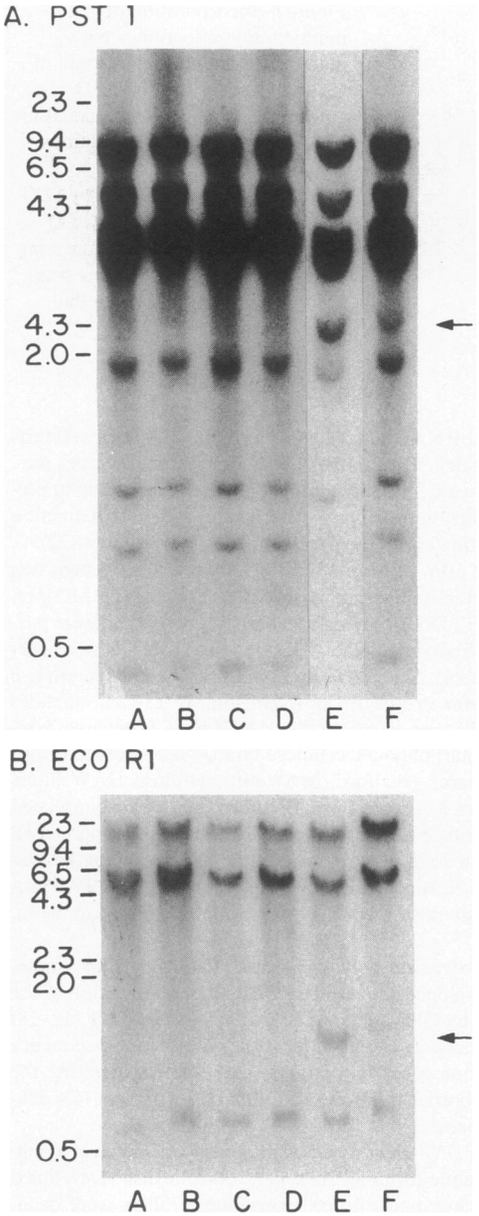


Figure 9. Southern blot analysis of Pst I (*A*) and Eco RI (*B*) digests of DNA from (*A*) normal outbred Hartley, (*B*) strain 2, (*C*) strain 13, (*D*) C2-deficient, (*E*) C3-deficient, and (*F*) C4-deficient guinea pigs. Additional 2.3 kb (Pst 1) and 1.6 kb (Eco RI) bands are visualized in the C3D and C4D lanes.

sulfate, 100 μg/ml, and hybridized in the same solution at 42°C using a radiolabeled ^{32}P-labeled mouse C3 cDNA probe kindly supplied by M. Nonaka and M. Takahashi. Filters were washed at 55°C in 1× SSC, 0.1% SDS for 1 h × 2, and subjected to autoradiography using Kodak XAR-5 film. Positive clones were purified by two subsequent rounds of plating and screening. After purification, candidate C3 clones were digested with Eco RI and subjected to 1% agarose gel electrophoresis.

cDNA sequencing. 10 μg of cDNA was self-ligated, sheared randomly by sonication (six bursts of 40 s), subcloned into M13mp18 according to methods described by Messing (25), and subjected to dideoxy-sequencing. In other experiments, double-stranded bluescript phagemids were denatured in 0.2 M NaOH, 1 mM EDTA for 5 min, ethanol precipitated, and then sequenced as above.

Construction of polymerase chain reaction (PCR) fragments. Oligonucleotides (29^{mers}) containing guinea pig C3 sequences with 5′ restriction endonuclease recognition sites were used to prime first strand cDNA synthesis from C3-deficient and C3-sufficient (Hartley) guinea pig liver mRNA from additional animals of each type. The RNA-DNA hybrid was amplified in a buffer containing 50 mM KCl, 35 mM Tris (pH = 8.3), 2.5 mM $MgCl_2$, bovine serum albumin (100 μg/ml), 1.25 mN dNTPs, sense and antisense primers (500 ng each) and 5 U Taq polymerase in a total volume of 100 μl. The reactions were carried out for 40 cycles of denaturation (2 min at 95°C), primer annealing (3 min at 61°C) and primer extension (7 min at 72°C), in an automated thermal cycler (Perkin Elmer-Cetus, Norwalk, CT). The product was subjected to electrophoresis on a 1% agarose gel (Seaplaque agarose; FMC Bioproducts, Rockland, ME), and the appropriate sized fragment isolated, phenol extracted and ethanol precipitated. The DNA was digested with the appropriate restriction enzyme, ligated into bluescript phagemid, and sequenced as above.

Results

C3 biosynthesis. Net biosynthesis of C3 was assessed in normal Hartley, C3D, and C4D guinea pig peritoneal cell cultures pulsed with [^{35}S]methionine for 4 h. The amount of radiolabeled pro-C3 (intracellular) and native C3 protein (extracellular) produced was quantitatively similar, and the apparent molecular mass of α chain (115 kD) and β chain (65 kD) were indistinguishable in cells from the normal and C3D guinea pigs (Fig. 1). In this experiment, pro-C3 produced by the C4D cells was apparently present in reduced amounts but the amount of extracellular native C3 was similar to that produced by the normal and C3D cultures. This decreased amount of pro-C3 in the C4D cells was not a reproducible finding since in four other experiments, the amount of pro-C3 synthesized in C4D cells was similar to that produced in normal and C3D cells. In each culture (normal, C4D, and C3D), the net incor-

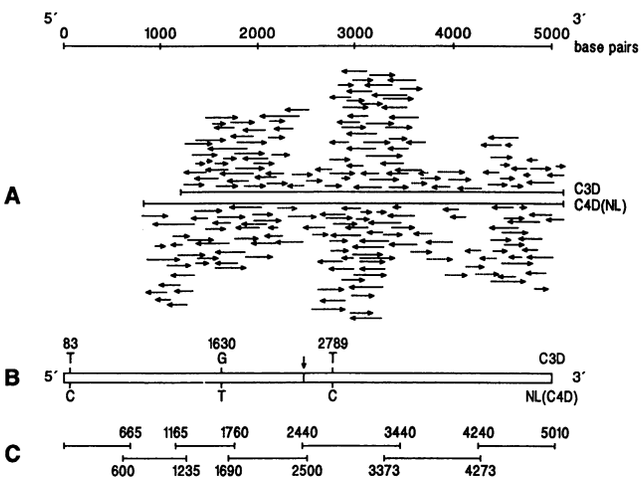


Figure 10. (*A*) Sequence strategy for C3D- and C3-sufficient (C4-deficient, C4D) C3 cDNA clones. Solid arrows represent individual gel readings from randomly sheared (shotgun) fragments. Dashed arrows represent additional sequence obtained from oligonucleotide-directed sequencing. (*B*) Solid bar represents sequence of C3D (*above*) and C3 sufficient (*C4D, below*) C3 cDNA, with base pair differences identified. Arrow indicates origin of primer extension libraries. (*C*) PCR-generated fragments derived from C3D and normal guinea pig liver mRNA for further sequence analysis.

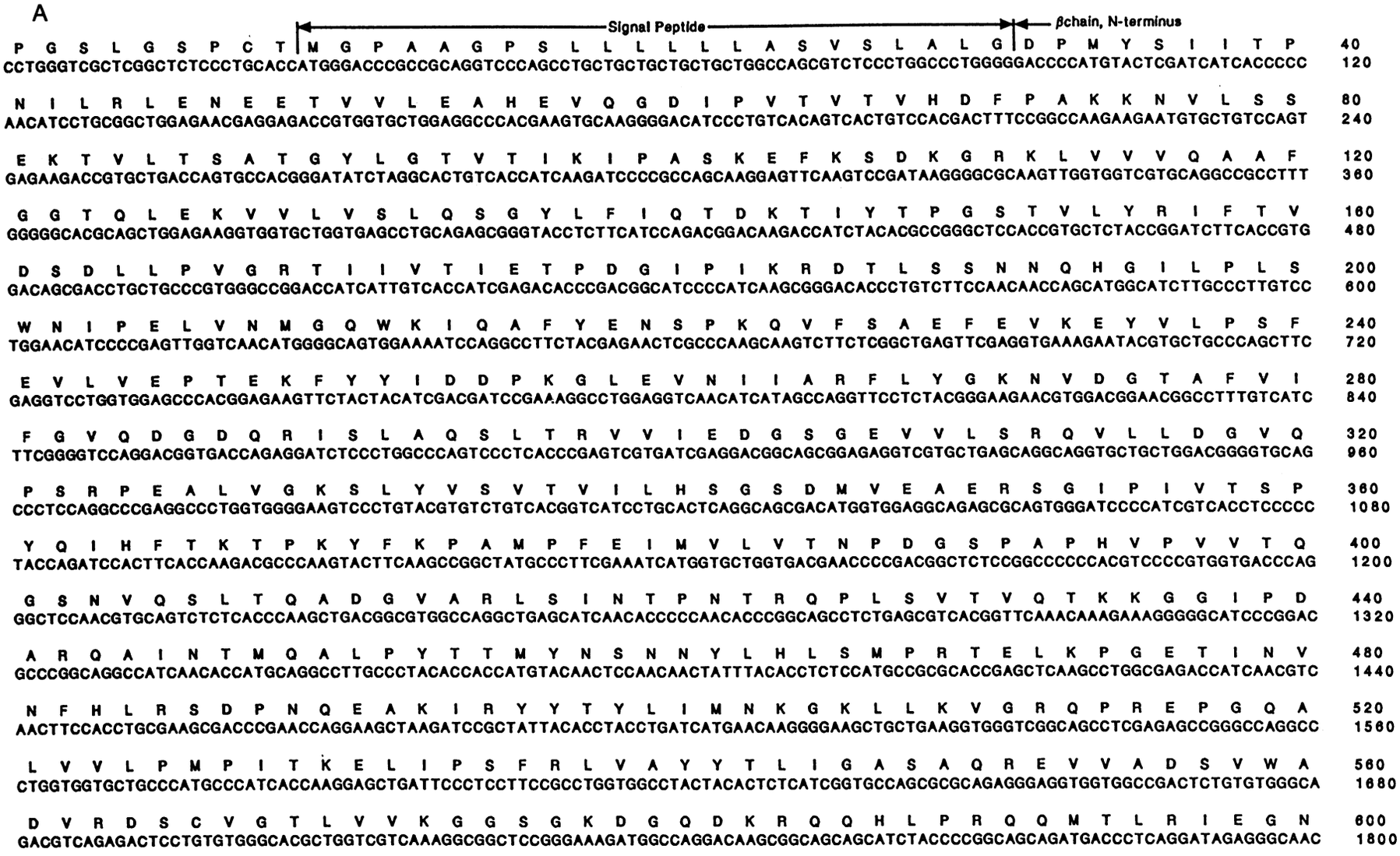
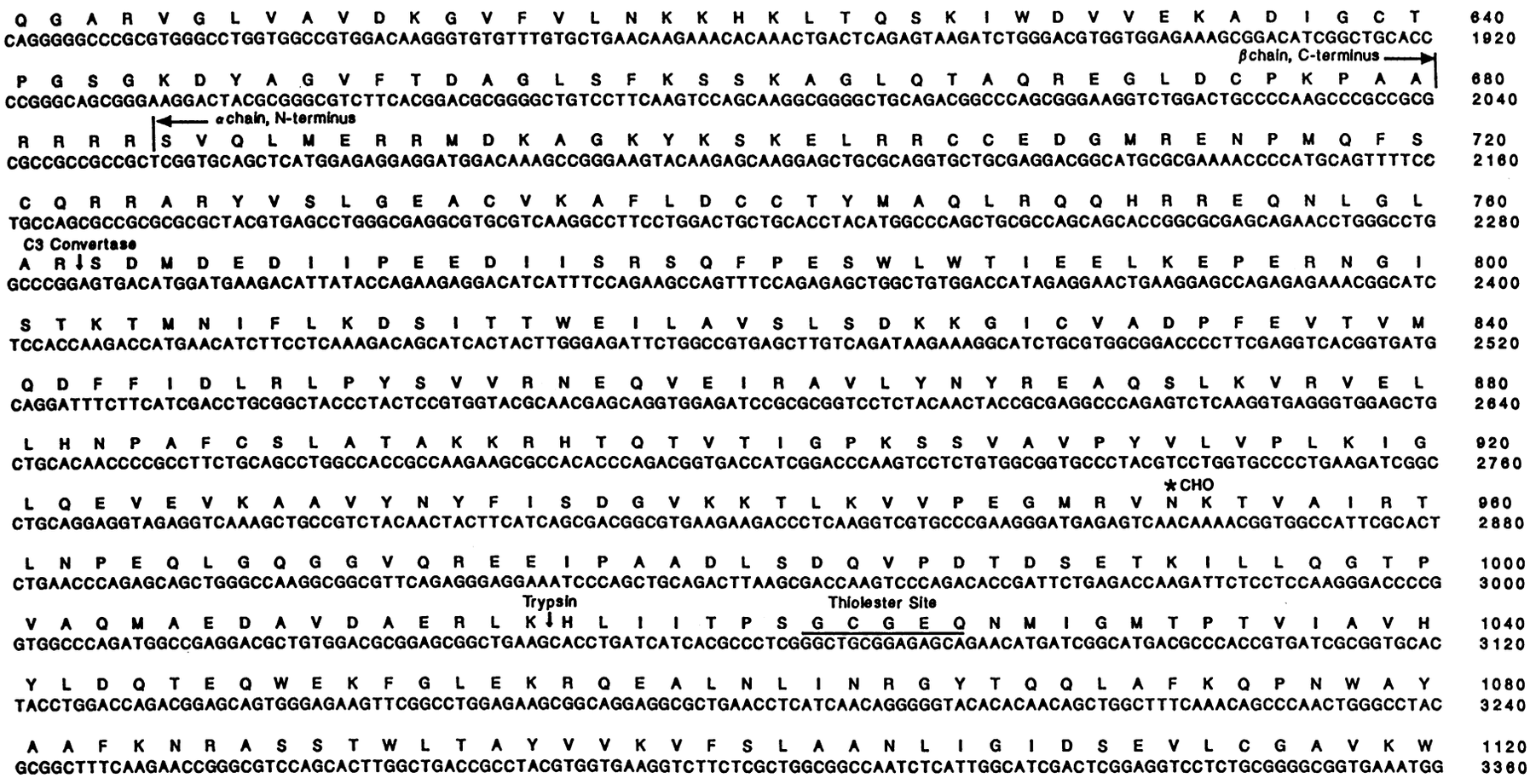


Figure 11. Complete nucleotide and derived amino acid sequences of guinea pig C3 cDNA. The signal peptide, NH_2 and COOH termini of the α and β chains are indicated. Also indicated are the thiolester region, asparagine-carbohydrate attachment sites, trypsin cleavage site, and Factor I cleavage site. These sequence data are available from GenBank under accession number M34054.

B



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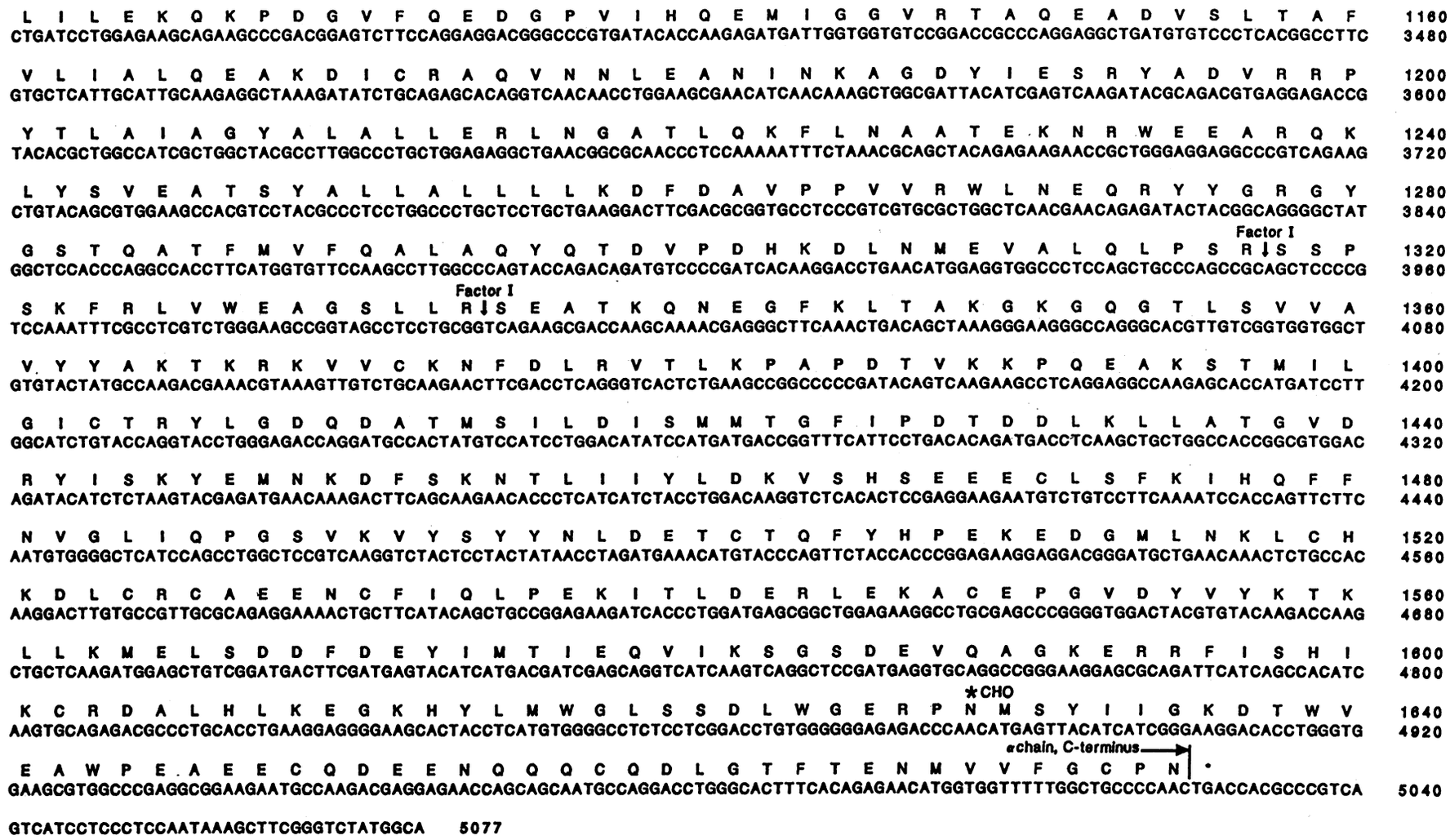

Figure 11 (Continued)

poration of [^{35}S]methionine in C3 of α chain was substantially greater than for β chain. Two additional polypeptides (~ 70 kD and 48 kD) were detected in the normal and C4D but not the C3D extracellular culture media (see below).

Isolated liver poly A$^+$ selected mRNA from C3D and normal Hartley guinea pigs generated comparable amounts of pro-C3 and secreted native C3 when injected into Xenopus oocytes pulse labeled for 72 h with [^{35}S]methionine (Fig. 2). The size and subunit composition of the C3D and normal guinea pig liver-derived native C3 were similar, but again the 70- and 48-kD products were only visualized when the oocytes were injected with normal mRNA (even overexposed gels failed to demonstrate 70- and 48-kD products in C3D-injected oocytes [data not shown]). Immunoprecipitation of C4, a complement protein homologous with C3, from the normal and C3D cultures (Fig. 3) revealed comparable amounts of pro-C4 intracellularly and native C4 in the extracellular medium.

Secretion of C3. To ascertain the rates of C3 secretion in normal and C3-deficient cells, peritoneal cell cultures were pulse labeled for 30 min with [^{35}S]methionine (500 μCi/ml), washed, and refed with medium containing unlabeled methionine. Cell lysates and media were then assayed for radiolabeled C3 protein (Fig. 4). These data showed that the kinetics of C3 secretion and net recovery of C3 protein were similar in the C3D and normal peritoneal cells.

Autolytic cleavage and thiolester bridge. Since the 70- and 48-kD polypeptides precipitable with antisera to C3 observed in Fig. 1 are similar in size to the previously reported C3 autolytic cleavage products, we attempted to generate these fragments from ^{35}S-labeled native C3 protein produced in C3D and normal peritoneal cell cultures by incubating the media in dilute alkaline SDS at 75°C for 20 min (21, 26). Again (Fig. 5), the 70- and 48-kD bands were visualized only in the normal but not in the C3D-derived culture media.

Methylamine incorporation into plasma C3, C4, and α_2 macroglobulin. Previous reports have shown that C3D guinea pig plasma contains C3 both functionally and antigenically at around 5% of the level found in normals. To examine the possibility that residual C3 in plasma from C3D guinea pigs differed from that produced in tissue culture the following experiment was performed. Plasma from C3D and normal guinea pigs were fractionated on an ion exchange column and aliquots of each fraction examined for the ability to incorporate ^{14}C-labeled methylamine, a function of an intact thiolester bridge. Fig. 6 demonstrates that methylamine was incorporated into α_2-macroglobulin and C4, two proteins that contain thiolester bonds, in both normal and C3D plasma; methylamine incorporation into C3 was detected only in normal but not C3D plasma even when the C3D plasma fractions were concentrated ~ 20-fold (data not shown) to account for the difference in content of C3 in the deficient plasma.

Proteolysis. Trypsin cleavage of native C3 protein produced by normal guinea pig peritoneal cells resulted in generation of C3b, as expected (Fig. 7). However, incubation of the C3D-derived native C3 protein with between 0.01 and 0.1 μg of trypsin (10-fold less than the minimal concentration required for C3b generation from normal C3) resulted in complete loss of immunoprecipitable C3 protein.

C3 transcription. As shown in Fig. 8, Northern blot analysis of total RNA isolated from C3D and C3 sufficient guinea pigs revealed mature C3 mRNA transcripts (~ 5 kb) comparable in size and amount. That is, C3 mRNA from normal and C3D guinea pig liver appeared qualitatively and quantitatively indistinguishable. The ethidium-stained gel indicated that comparable amounts of RNA were analyzed.

C3 gene structure. DNA was isolated from Hartley, C2-deficient (strain 13), C4-deficient (strain 13), and C3-deficient (strain 2) animals, digested with several restriction enzymes (Pst I, Eco RI, Bam HI, Hind III, Bgl I, and Sma I) and subjected to Southern blot analysis (27) using a full length normal guinea pig C3 cDNA probe. Fig. 9 shows that digestion with either Pst I or Eco RI resulted in restriction fragment length polymorphisms (additional 2.3 kb [Pst I] and 1.6 kb [Eco RI] bands) in the C3D and C4D samples. Since C3 protein synthesis and C3 serum concentrations are normal in C4-deficient guinea pigs, this polymorphism is not a marker for the C3 deficiency phenotype.

Comparison of C3 cDNA from C3-sufficient and deficient liver libraries. 17 C3 clones from the C3-deficient library and 25 clones from the C4-deficient library were detected; the largest insert, 3.8 kb in the C3-deficient and 4.2 kb in the C4-deficient libraries. Inserts from the three largest clones of each library were isolated and sequenced. As shown in Fig. 10, ~ 70% of the cDNA was analyzed using this sequencing strategy, with most regions sequenced several times on both strands. To complete the sequence, oligonucleotides were constructed and used as primers for dideoxy sequencing directly from phagemids. Using this initial strategy, the C3 cDNA sequence extended from the poly(A) tail through the 750 and 1150 most 5′ nucleotides of the β chain for C3-deficient and C3-sufficient clones, respectively.

To complete the C3 cDNA sequence, primer extension cDNA libraries were constructed using a C3 specific oligonucleotide (20 mer) corresponding to positions 2469–2488 of complete guinea pig C3 cDNA sequence. Clones isolated from each library contained sequences that extended 29 nucleotides 5′ of the C3 signal peptide and overlapped by 1312 and 1712 nucleotides, the largest clones from the C3-deficient and C3-sufficient libraries. Confirmation of the normal and C3 deficient guinea pig C3 sequence was accomplished by direct sequence analysis of PCR fragments generated from C3 sufficient (Hartley) and C3 deficient mRNA (Fig. 10).

The complete C3-sufficient (C4 deficient) guinea pig C3 cDNA sequence and derived amino acid sequence are shown in Fig. 11. The sequence spans 5081 nucleotides, from 29 base pairs upstream of the signal peptide to the beginning of the poly(A) tail. A putative polyadenylation signal, AATAAA, is located 16 nucleotides upstream from the polyadenylation site, and is identical to the polyadenylation signal described for murine C3 and Slp. The deduced C3 protein sequence consists of 1666 amino acids in a β-α chain orientation with four arginines interposed between the β and α chains. The coding region is, therefore, equal in size to murine C3 and three amino acid residues longer than human C3. Overall, 77–80% nucleotide and amino acid identity was observed for human, mouse, and guinea pig C3. Complete nucleotide identity between human and guinea pig C3 sequences was found at the thiolester 15-base pair segment within the flanking 45 base pairs in each direction. Guinea pig C3 shares other structural and functional characteristics with human and murine C3, including two asparagine carbohydrate attachment sites at positions

944 and 1620, a trypsin cleavage site between lysine at position 1006 and histidine at 1007, and two Factor I cleavage sites at positions 1310 and 1327.

C3-sufficient (C4 deficient) and C3-deficient guinea pig C3 cDNA sequences were compared. Only three nucleotide differences, at positions 83, 1630, and 2789 were found (Fig. 10). These nucleotide differences are "silent" in that they do not result in a difference in the derived amino acid sequences. The sequence of PCR fragments from an additional C3-deficient liver mRNA was identical to the sequence generated from the C3-deficient cDNA and primary extension libraries. The Hartley C3 PCR fragments showed identity with the other C3-sufficient (C4 deficient) C3 cDNA sequence except at positions 83 (T to C); i.e., a substitution identical at this position with the C3-deficient sequence.

Discussion

Several possible explanations were considered to account for discrepancies between serum concentration of C3 and rate of C3 biosynthesis in previous studies of humans with genetically determined C3 deficiency (1–11). However, those experiments necessarily monitored C3 biosynthesis only at an extrahepatic site (blood monocytes) since ethical considerations precluded a direct test of the hypothesis that C3 synthesis rate in liver was more closely related to the deficiency than C3 synthesis in other tissues. One possibility that was not considered at that time was that a defective C3 protein susceptible to accelerated catabolism was produced by the deficient cells even though the newly synthesized C3 protein appeared grossly normal by estimates of size and subunit composition. Moreover, a near normal rate of catabolism of normal purified radiolabeled C3 protein was observed in a C3-deficient patient (3). Similar findings were obtained in the initial studies of C3 catabolism in C3-deficient guinea pigs (14). Preliminary data from those studies also suggested that C3 synthesis is normal in C3-deficient guinea pig hepatocytes.

In the present report we established that liver and peritoneal cells from C3-deficient, normal, and C4-deficient guinea pigs (*a*) contain C3 specific mRNA indistinguishable in size and quantity by Northern blot analysis, and (*b*) synthesize pro-C3 and secrete native C3 protein at similar rates. Although the C3 protein produced by the C3D peritoneal cells or secreted by oocytes primed with C3D liver mRNA appears normal in size and subunit composition it differs from normal C3 in at least two respects. That is, the C3D-derived C3 protein fails to undergo autolytic cleavage and is unusually susceptible to proteolytic digestion.

C3, C4, and α_2 macroglobulin are proteins that share an unusual structural feature, a thiolester bridge, important in their respective functions. The phenomenon of autolytic cleavage in dilute alkaline SDS is associated with the presence of an intact thiolester bridge. The C3 protein produced in C3-deficient guinea pigs, by this criterion, appears to display a defect in the thiolester bridge or its accessibility. Support for this concept and for the selectivity of the defect is provided by demonstrating incorporation of methylamine into C4 and α_2 macroglobulin, but not into C3 of C3 deficient plasma even when the samples are concentrated ~ 20-fold to increase the C3 content in the C3D sample to near normal levels. An abnormality in primary or higher order structure of the C3-deficient C3 protein is also suggested by the unusual susceptibility of this protein to proteolysis. The rate of in vivo catabolism of this abnormal C3 protein is unknown because previous estimates of C3 metabolism in the deficient animals as in studies of C3D humans were obtained using normal purified radiolabeled C3 protein (14).

These data are consistent with (*a*) a mutation in the coding region of the C3 gene leading to a defect in primary or higher order structure affecting the thiolester bridge or its accessibility, (*b*) a C3 specific co- or post-translational processing defect that affects the thiolester bridge; i.e., a genetic defect unlinked to the C3 gene. The finding of a restriction fragment length polymorphism (RFLP) that does not correlate with the C3 deficiency phenotype lends support to the latter hypothesis. That is, the RFLP provides a marker for the C3 structural gene distinct from the deficiency phenotype. Sequence analysis of C3 cDNA from C3 deficient guinea pig liver libraries ruled out a mutation in primary structure of the C3 gene as the basis for this genetic defect.

Sequencing of C3 cDNA clones from two separate C3 deficient liver libraries and from PCR fragments generated from a third C3-deficient liver mRNA preparation established that the C3 coding regions in C3-sufficient and deficient guinea pigs is identical except for positions 83, 1630, and 2789. None of these substitutions result in an amino acid difference. At position 83 the Hartley (C3 sufficient) sequence is identical to the C3-deficient sequence. These data thus support the hypothesis that C3 deficiency in guinea pig is due to a co- or postsynthetic processing modification that renders the C3 protein highly susceptible to proteolysis.

The biochemical mechanism by which the thiolester bridge is generated in C3 is uncertain. Kahn and Erickson (28) suggested that isomerization of a lactam ring to generate the thiolactone proceeds spontaneously under physiological conditions, though they could not rule out an enzymatically facilitated process. Iijima et al. (29) on the other hand provided tentative evidence for participation of a cytoplasmic factor (possibly an enzyme) in the formation of the C3 thiolester. Heretofore, investigators have assumed but not tested the assumption that the mechanisms for generation of the C3, C4 and α_2 macroglobulin thiolesters are identical. Since the mutation accounting for C3 deficiency is not in the coding region of the C3 gene, this defect provides a probe to examine co- and postsynthetic processing of this important group of proteins. Molecular biological and classical genetic studies are underway to test this hypothesis.

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