Identification and Structural Characterization of Two Incompletely Processed Forms of the Fourth Component of Human Complement

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ABSTRACT Immunoprecipitates of human C4 from EDTA-plasma were incubated with [^{14}C]methylamine and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and fluorography. In addition to finding label in the α-chains of the secreted ($C4^s$) and predominant plasma ($C4^P$) forms of C4, two additional molecules with apparent molecular weights of ~168,000 (p168) and ~125,000 (p125) covalently incorporated methylamine, indicating the presence of an internal thioester bond. These two molecules were present at a concentration of ~5% of total plasma C4 and were not immunoprecipitated by antisera to C3 or α_2-macroglobulin. A human hepatoma-derived cell line (HepG2), in addition to synthesizing $C4^s$ and small quantities of the polypeptide precursor of C4 (pro-C4), was found to secrete p168 and p125 at concentrations of 14±4.8 and 21±9.2% (mean±SD), respectively, of total secreted C4. These molecules were not found intracellularly. Both molecules were present on reduced, but not nonreduced, SDS-polyacrylamide gels. Chido (C4B) and Rodgers (C4A) alloantisera precipitated the C4A and C4B variants of pro-C4, p168, p125, and $C4^s$. Both tryptic and *Staphylococcus aureus* V8 protease peptide analyses showed homology between p168 and the β- and α-chains and between p125 and the α- and γ-chains. Partial NH_2-terminal sequencing revealed that the β-chain was NH_2-terminal in p168 and that the α-chain was NH_2-terminal in p125. Taken together, these data indicate that p168 and p125 represent uncleaved β-α- and α-γ-fragments of pro-C4, respectively. Thus, in most individuals, plasma C4 consists of five structurally distinct molecules, the single polypeptide precursor (pro-C4), the three-subunit secreted ($C4^s$) and predominant plasma ($C4^P$) forms of C4, and two incompletely processed two-subunit molecules with uncleaved β-α- (p168) or uncleaved α-γ (p125)-subunits. In addition, all five molecules are observed for both C4A (Rodgers) and C4B (Chido) structural genes.

INTRODUCTION

The fourth component of complement (C4)[1] is an ~200,000-D glycoprotein consisting of three polypeptides, designated α, β, and γ, linked together by disulfide bonds (1). C4 is synthesized as pro-C4, a single-chain polypeptide, and is processed by intracellular cleavage to yield the secreted three-chain C4 molecule (2–4). The order of the subunits in mouse and guinea pig pro-C4 has been determined to be β-α-γ (5–7). We have previously described and characterized human and mouse $C4^s$, the secreted form of C4, which has an α^s-chain with an apparent molecular weight ~5,000 greater than the α^P-chain of $C4^P$, the predominant

This work was presented, in part, at the Annual Meetings of the Federation of American Societies for Experimental Biology, Chicago, IL, March 1983, and of the American Association of Physicians, Washington, DC, April 1983, and published in abstract form in 1983. *Fed. Proc.* 42:841*a*. (Abstr.); *Clin. Res.* 31:538*a*. (Abstr.)

Received for publication 27 April 1983 and in revised form 11 July 1983.

[1] *Abbreviations used in this paper:* α_2M, α_2-macroglobulin; α^P, α-chain of $C4^P$ with M_r of 93,000; α^s, α-chain of $C4^s$ with M_r of 98,000; bis, *N,N'*-methylene bisacrylamide; C3 and C4, third and fourth components of complement; C4b, C4 activation fragment consisting of the β- and γ-chains and the COOH-terminal portion of the α-chain; $C4^P$, principal plasma form of C4; $C4^s$, secreted form of C4; HepG2, hepatoma-derived cell line; HPLC, high-performance liquid chromatography; MEM, minimum essential medium; PAGE, polyacrylamide gel electrophoresis; p125, 125,000-D polypeptide seen on reducing gels representing the uncleaved α-γ-subunits of pro-C4; p168, 168,000-D polypeptide seen on reducing gels representing the uncleaved β-α-subunits of pro-C4.

plasma form of C4 (8, 9). $C4^s$ constitutes ~8% of the total C4 found in human or mouse plasma and is converted extracellularly, presumably by proteolytic processing, to $C4^p$. In our studies of the $C4^s$ molecule found in human plasma and that synthesized by a human hepatoma-derived cell line (HepG2), two additional C4-related molecules having M_r of ~168,000 and ~125,000 were detected on fluorographs of C4 immunoprecipitates analyzed under reducing conditions. In this report, we demonstrate that these two molecules represent incompletely processed two-subunit C4 fragments in which only one of the two intracellular cleavages of pro-C4 has occurred.

METHODS

Reagents

The IgG fraction of goat anti-human C4, anti-α_2-macroglobulin (α_2M), and anti-human C3 antisera were purchased from Atlantic Antibodies (Scarborough, ME). Radiochemicals used were [^{35}S]methionine (1,026 Ci/mmol), L-[4,5-^{3}H]leucine (130 Ci/mmol), L-[3,4(*n*)-^{3}H]valine (30 Ci/mmol), and L-[4,5-^{3}H]lysine (80 Ci/mmol) from Amersham Corp. (Arlington Heights, IL), and D-[2(*n*)-^{3}H]mannose (130 Ci/mmol) and [^{14}C]methylamine (51.8 mCi/mmol) from New England Nuclear (Boston, MA) (1 Ci = 3.7×10^{10} becquerels). *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories Inc., Research Products Div. (Elkhart, IN). Anti-Chido and anti-Rodgers alloantisera (10) were a generous gift from John Moulds (Gamma Biologicals, Houston, TX). All other chemicals were of reagent grade from standard sources.

Cells and biosynthetic labeling

HepG2, a human hepatoma-derived cell line, was obtained from the Wistar Institute of Anatomy and Biology (Philadelphia, PA) (4, 11). Conditions for culturing and biosynthetic labeling with [^{35}S]methionine, L-[4,5-^{3}H]leucine, L-[3,4(*n*)-^{3}H]valine, or L-[4,5-^{3}H]lysine in methionine, leucine, valine, or lysine-free minimal essential medium (MEM), respectively, have been described (8). Labeling with D-[2(*n*)-^{3}H]mannose was accomplished in serum-free MEM. Labeled medium was collected and either used immediately or frozen at −70°C for later use.

To obtain the intracellular fraction, cells radiolabeled for 2 h were washed with cold MEM supplemented with 10 mM EDTA and incubated in 10 mM EDTA-MEM for 10 min at 4°C. Cells were centrifuged at 1,000 *g* at 4°C and the cell pellet homogenized in 2 ml of 2 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 2% Triton X-100, 5 mM EDTA, 20 mM Tris (pH 8.0) with a Dounce homogenizer. The intracellular fraction was then collected by centrifugation at 100,000 *g* for 1 h at 4°C and frozen at −70°C for later use.

Immunoprecipitation and immunoadsorption

C3, C4, and α_2M were immunoprecipitated from plasma at equivalence. Labeled C4 was immunoprecipitated from hepatocyte medium in the presence of carrier plasma, 1 mM phenylmethylsulfonyl fluoride, 0.5 M KCl, and 10 mM EDTA, washed, and prepared for electrophoresis and fluorography (6). C4 was also purified by incubating labeled hepatocyte medium with anti-C4 antisera immobilized on a protein A-Sepharose immunoadsorbent (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, NJ) (12).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography

SDS-PAGE on gradient slab gels and 5, 7.5, and 9% polyacrylamide slab gels with both reducing (15% [wt/vol] β-mercaptoethanol, 6% [wt/vol] SDS, 0.75 M Tris, pH 6.8) and nonreducing conditions was performed according to the method of Laemmli (13). Preparation of a linear gradient from 6 to 18% polyacrylamide with a 3% stacking gel, fixation, staining, destaining, and fluorography have been described (14). Before drying, destained gels were treated with EN^{3}HANCE (New England Nuclear). For separation of the α-chains of C4 encoded by C4A (Rodgers) and C4B (Chido) genes, 10% polyacrylamide gels with an acrylamide/*N*,*N*′-methylene bisacrylamide (bis) ratio of 1:0.006 as described by Roos et al. (15) was used. For densitometric studies, Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) was preexposed in an electronic photographic flash unit with a colored filter (16). Quantitation of fluorographs utilized a Zeineh soft laser scanning densitometer (Biomed Instruments Inc., Chicago, IL) or a Kontes model 800 fiberoptic scanner (Kontes Scientific Instruments, Vineland, NJ) in the transmission mode (17). For molecular size calibrations, eight standard proteins (Bio-Rad Laboratories, Richmond, CA) were used: myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Structural analysis of C4

Radiolabeling of internal thioester bond with [^{14}C]methylamine. Immunoprecipitates were resuspended in veronal-buffered saline, pH 8.0, and incubated with ~4 mM [^{14}C]methylamine for 4 h at 37°C (18). The methylamine-treated immunoprecipitates were then washed three times with 10 mM EDTA, pH 7.4, and prepared for electrophoresis.

Tryptic peptide analysis. [^{3}H]Leucine- or [^{35}S]methionine-labeled proteins eluted from SDS-polyacrylamide gels were mixed with 1 mg carrier bovine gamma globulin, reduced with 2.5 mg/ml dithiothreitol, and alkylated with 9 mg/ml iodoacetamide (19). Protein was precipitated overnight at 4°C with 20% trichloroacetic acid (TCA), washed twice with cold 5% TCA acid, once with 95% ethanol/anhydrous ether (1:1), and twice with cold anhydrous ether. Residual ether was removed by evaporation and the protein resuspended in 1 ml 0.1 M ammonium bicarbonate. Proteins were digested with 0.1 mg L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemicals Corp., Freehold, NJ) over a 14-h period at 37°C with constant shaking. Equal aliquots of fresh TPCK-trypsin were added after 1 and 13 h of incubation. Digests were lyophilized and prepared for high-performance liquid chromatography (HPLC) analysis by resuspension of the peptides in 0.5 ml 0.01 M tetrabutylammonium phosphate and centrifugation to remove insoluble material.

Peptide separation by HPLC was performed as previously described (20) with a 4 × 250 mm-column of LiChrosorb RP-8 (E. Merck, Darmstadt, FRG) in a Spectra-Physics Inc. (Mountain View, CA) SP8000B liquid chromatograph equipped with a microprocessor-controlled ternary gradient marker and an optical density monitor. The peptides were resolved on a gradient of 0 to 75% acetonitrile in 0.01 M aqueous tetrabutylammonium phosphate, pH 7.0. Fractions (0.25 ml) were collected and counted for radioactivity. The 220-nm OD profile of BGG tryptic peptides was used as an internal standard.

S. aureus V8 protease peptide analysis. Peptide mapping by limited proteolysis utilizing *S. aureus* V8 protease was performed as described by Cleveland et al. (21). C4 immunoprecipitates of [^{35}S]methionine-labeled hepatocyte medium were reduced and alkylated and proteins separated by SDS-PAGE. Bands were cut from the gel and digested by placing gel slices containing the protein in the sample wells of a second SDS-polyacrylamide gel and then overlaying each slice with 0.5 μg of *S. aureus* V8 protease. Electrophoresis was performed until the bromophenol blue dye neared the bottom of the stacking gel, when the current was turned off for 30 min. The peptides were then separated on a 15% polyacrylamide running gel and analyzed by fluorography.

NH_2-terminal sequencing. [^{3}H]Leucine-, [^{3}H]valine-, or [^{3}H]lysine-labeled proteins eluted from SDS-polyacrylamide gels were mixed with 100 nmol carrier sperm whale apomyoglobin, dialyzed extensively in the presence of AG1-X2 anion exchange resin (Bio-Rad Laboratories) to remove SDS, and lyophilized for sequencing. NH_2-Terminal sequencing with a Beckman 890C Sequencer (Beckman Instruments, Inc., Fullerton, CA) was done by Dr. Gregory Grant (Department of Biological Chemistry, Washington University School of Medicine). Polybrene (3 mg) was added before each run and a 0.33 M Quadrol program was used (22). A blank cycle was used at the beginning of each cycle to remove nonspecific radioactivity. The thiazolinone derivatives were extracted with butyl chloride, dried, redissolved in 1 ml methanol, and added to 10 ml 3A70 scintillation fluid for counting radioactivity. Sperm whale apomyoglobin (100 nmol) was used as an internal standard. Sequencer repetitive yield was >99%.

RESULTS

p125 and p168 are antigenically related to C4 and possess the internal thioester bond. In our characterization of the C4^s molecule (8), fluorographs of anti-C4 immunoprecipitates from human plasma incubated with [^{14}C]methylamine revealed, in addition to the α^s- and α^p-chains, two molecules with approximate M_r of 168,000 (p168) and 125,000 (p125). Densitometric scanning of the fluorographs indicated that p168 and p125 together accounted for ~5% of the [^{14}C]methylamine bound to C4^s and C4^p. Additional anti-C4 immunoprecipitates of EDTA-plasma from 10 normal individuals were incubated with [^{14}C]-methylamine and analyzed by SDS-PAGE and fluorography (Fig. 1). Both p168 and p125 were present in all 10 individuals, although p125 present in smaller quantities made visualization difficult upon photographic reproduction. In other individuals, however, p125 is the predominant molecule of the two (Fig. 2).

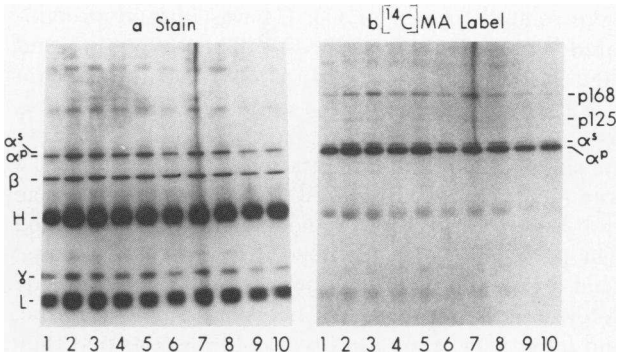

FIGURE 1 Presence of p168, p125, C4^s, and C4^p in human plasma. (*a*) Commassie Brilliant Blue protein stain of C4 immunoprecipitate from EDTA-plasma of 10 normal individuals analyzed on a 6–18% gradient slab gel under reducing conditions. In most tracks, lightly stained bands with M_r of ~190,000, ~150,000, and ~50,000 are seen. These proteins do not incorporate methylamine and their identity is unknown. The heavy (H) and light (L) chains of the anti-C4 antisera can also be seen. (*b*) Corresponding fluorograph of these C4 immunoprecipitates incubated with 4 mM [^{14}C]methylamine (MA). Radiolabel is seen in α^s, α^p, p168, and p125. Radioactivity was also detected in bands comigrating with the H and L chains. The amount present, however, was not in proportion to the quantity seen on the protein stain.

Three molecules, α_2M and the α-chains of C3 and C4, are known to incorporate methylamine (18, 23, 24). To evaluate the possibility that p168 and p125

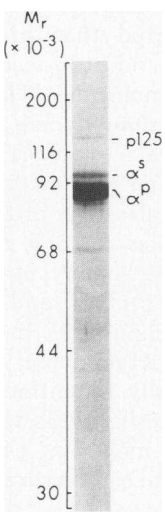

FIGURE 2 Predominance of p125 in the plasma of some individuals. As in Fig. 1, C4 was purified from EDTA-plasma, incubated with [^{14}C]methylamine, and analyzed by electrophoresis (9% polyacrylamide gel) and fluorography. The fluorograph is shown. In this overexposed gel some nonspecific trapping of radioactivity is seen in a molecule with a M_r of ~68,000, presumably human serum albumin. In contrast to p125 and the α-chains, radioactivity in this 68,000-D band was not in proportion to quantity of protein seen on the protein stain (not shown).

were related to α_2M or C3, C4 was immunoprecipitated from serum, labeled with [^{14}C]methylamine, and their M_r compared. The M_r of α_2M and the α-chain of C3 were ~185,000 (25) and ~110,000 (26), respectively, and were distinct from p168 and p125 (data not shown). Immunoprecipitation of α_2M, C3, and C4 from a human hepatoma cell line known to synthesize and secrete these three molecules (27) also revealed that p168 and p125 are not C3 or α_2M (see below). That p125 and p168 (*a*) possess common antigenic determinants with C4, (*b*) incorporate methylamine, and (*c*) are not related to C3 or α_2M suggests that they are C4-related peptides with an intact thioester bond in their α-chains. An incompletely processed precursor fragment of murine C4 has been described (20). Thus, p168 and p125 are likely candidates for human C4 processing fragments. Furthermore, the M_r suggest that p168 and p125 are C4-related molecules with uncleaved $\beta + \alpha$- and $\alpha + \gamma$-chains, respectively. The small quantities of material present in plasma, however, make definitive identification difficult. Hence, a human hepatoma cell line capable of synthesizing C4 was used to study these putative C4 molecules further.

Synthesis of p168 and p125 by HepG2. During our characterization of the C4^s molecule (8), we noted that HepG2 synthesized and secreted, in addition to C4^s, significant quantities of C4 molecules corresponding to p125 and p168 found in plasma (Figs. 3 *a* and 4). Quantitation by densitometric scanning (mean of five experiments) indicated that p168 accounted for 14±4.8% (mean±SD) and p125 accounted for 21±9.2% of the secreted C4 molecules. These two molecules were not present on nonreduced gels (Fig. 3 *b*), nor were they found intracellularly (Figs. 4 and 5). Furthermore, both p168 and p125 incorporate [^{3}H]mannose (Fig. 5, lane *2*), indicating the presence of sugar moieties on both molecules. C4 is a glycoprotein with a complex carbohydrate moiety on the α-chain, high mannose units on the β-chain, and minimal or no carbohydrate on the γ-chain (29). In these experiments, no incorporation of [^{3}H]mannose was detected in the γ-chain (Fig. 5). Finally, the intracellular pro-C4 molecule migrated with a slightly faster mobility than the extracellular pro-C4 molecule (Figs. 4 and 5). This may reflect incomplete carbohydrate processing of the pro-C4 molecule.

Immunoprecipitation of α_2M, C3, and C4 from labeled hepatocyte medium confirmed that p168 and p125 were not α_2M or C3 (Fig. 6). Sequential immunoadsorption of α_2M, C3, and finally C4 revealed similar results (data not shown). Hence, despite having M_r similar to p168 and p125, α_2M and the α-chain of C3 are different electrophoretically as well as antigenically.

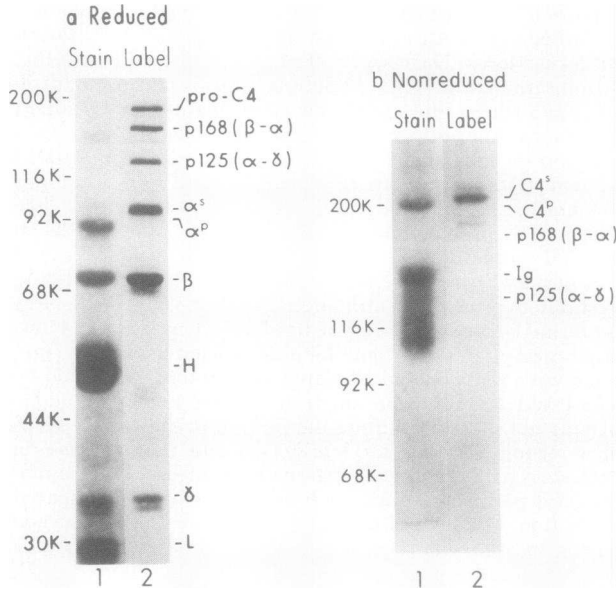

FIGURE 3 Secretion of pro-C4, p168, p125, and C4^s by HepG2 cells. (*a*) C4 immunoprecipitate of HepG2 extracellular media. HepG2 cells were biosynthetically labeled with [^{3}H]leucine (200 μCi/ml) for 4 h and the extracellular media immunoprecipitated with anti-C4 antisera in the presence of carrier plasma as described in Methods. The C4 immunoprecipitates were analyzed by electrophoresis (7.5% polyacrylamide gel) and fluorography. Lane *1*, protein stain; lane *2*, fluorograph. As previously described (8), the labeled α^s-molecule does not correspond to the protein staining of the α^p-chain. In addition to the α^s-chain, three proteins with M_r of 185,000, 168,000, and 125,000 were also secreted by the HepG2 cells. The 185,000-D molecule corresponds to pro-C4, while the 168,000- and 125,000-D molecules appear to correspond to p168 and p125 found in plasma. The γ-chain of C4 immunoprecipitated from serum and hepatocyte culture migrates as a doublet (under reducing conditions with 15% β-mercaptoethanol) with an ~2,000-D difference. Upon reduction with dithiothreitol and alkylation with iodoacetamide, however, only a single band corresponding to the lower band is observed (not shown). Gigli et al. (29) have demonstrated that the γ-chain has three times the number of cysteine residues per 100 amino acids than the α- and β-chains. Partial dissociation and association of proteins with an unusually high cysteine content (e.g., wheat germ agglutinin, reference 28) have been described and the C4 γ-chain may exhibit this same phenomenon. (*b*) C4 immunoprecipitate of HepG2 extracellular media analyzed by electrophoresis (5% polyacrylamide gel) and fluorography under nonreducing conditions. C4^s (lane *2*) has an apparent molecular weight ~5,000 greater than C4^p (lane *1*). The markers for p168 and p125 denote where these proteins would have migrated on the basis of the molecular weight standards. Also present in lane *2* are three minor bands in the 180,000-D range, which make up <5% of the total counts. The identity of these bands is unknown.

To study the kinetics of p168 and p125 secretion, HepG2 cells were pulsed for 2 h with [^{35}S]methionine and then chased with cold medium (Fig. 7). The two

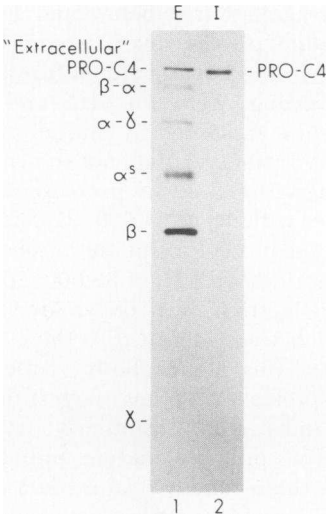

FIGURE 4 Comparison of C4 molecules found intracellularly and extracellularly in HepG2 cultures. HepG2 cells were labeled with [^{35}S]methionine and extracellular and intracellular fractions were immunoadsorbed with anti-C4 antibody immobilized on protein A-Sepharose. Immunoadsorbed C4 was eluted and analyzed under reducing conditions by electrophoresis (9% polyacrylamide gel) and fluorography. While C4^s, β-α, and α-γ were found extracellularly (lane *1*), only pro-C4 was detected intracellularly (lane *2*). The intracellular pro-C4 molecule has a M_r ~1,000 less than the extracellular pro-C4 molecule. This may be due to incomplete carbohydrate processing of the intracellular pro-C4 molecules.

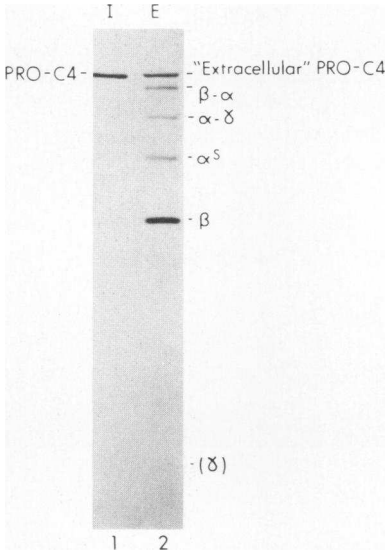

FIGURE 5 Comparison of [^{3}H]mannose-labeled C4 molecules found intracellularly and extracellularly in HepG2 cultures. HepG2 cells were biosynthetically labeled with [^{3}H]mannose and C4 purified from the extracellular and intracellular fraction by immunoadsorption as described in Methods. Purified C4 was then analyzed by electrophoresis (9% polyacrylamide gel) and fluorography. As in Fig. 4, C4^s, β-α, α-γ, and extracellular pro-C4 were purified from the extracellular fraction (lane *1*), while only pro-C4 was detected intracellularly (lane *2*). The γ-chain did not incorporate detectable mannose and the marker for the γ-chain denotes where it would have migrated on the basis of the molecular weight markers.

molecules were secreted concurrently and densitometric scanning at 2, 4, and 6 h postchase indicated that the ratios of C4^s/p168/p125 were similar throughout the chase period. These data indicate that for up to 6 h after secretion, p168 and p125 are neither further processed in the hepatocyte cultures nor derived by extracellular proteolytic cleavage of pro-C4. Pulse-chase experiments with shorter (30 min or 1 h) pulse periods revealed similar data.

Tryptic peptide analysis and S. aureus V8 protease analyses. To elucidate further the structure of p125 and p168, the two molecules were analyzed by tryptic peptide analysis. p125, p168, and C4 α^s-, β-, and γ-chains were purified by elution from SDS-polyacrylamide gels of C4 immunoprecipitates of labeled hepatocyte medium, digested with trypsin, and the tryptic peptides separated by HPLC. Fig. 8 shows the tryptic peptide maps of [^{35}S]methionine labeled C4 α^s, C4 β, and p168. All of the major peaks of the α^s- and β-chain peptides were present in the p168 peptide map. Unshared minor peptides may be due to the COOH-terminal peptide of the β-chain, the NH_2-terminal peptide of the α^s-chain, and, if the β-chain does not have a lysine or arginine COOH-terminus, the

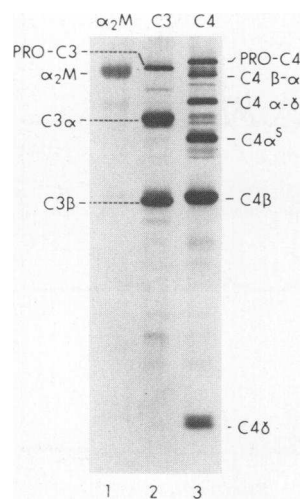

FIGURE 6 p168 and p125 are unrelated to α_2M or the α-chain of C3. Anti-α_2M, anti-C3, or anti-C4 immunoprecipitates of labeled hepatocyte medium were analyzed by electrophoresis (9% polyacrylamide gel) and fluorography. The fluorograph of α_2M (lane *1*), C3 (lane *2*), and C4 (lane *3*) immunoprecipitates from labeled HepG2 medium is shown.

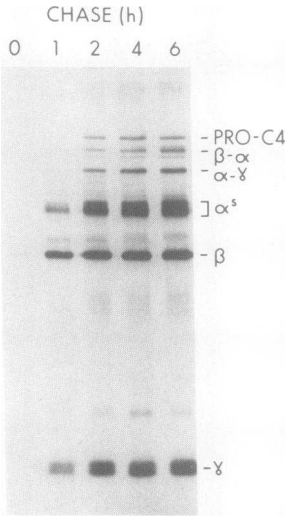

FIGURE 7 Secretion of C4 by HepG2. HepG2 cells were pulsed 2 h with 200 μCi/ml of [^{35}S]methionine in 5 ml of methionine-free MEM and then chased for 0, 1, 2, 4, and 6 h in unlabeled methionine containing MEM. C4 was immunoprecipitated from 1-ml aliquots taken at each interval and analyzed on a 9% polyacrylamide gel. The α^s-chain sometimes migrates as a broad band and probably reflects α-chain heterogeneity.

combination of these two peptides in p168. The 5,000-D COOH-terminal fragment of α^s may account for further differences if p168 is a β-α^p-fragment, rather than a β-α^s-fragment (see below and Fig. 12). This possibility is unlikely, however, since C4^s is not converted to C4^p in the hepatocyte cultures (Fig. 3).

Peptide homology between p168 and the α^s- and β-chains was also examined by limited proteolysis using *S. aureus* V8 protease (data not shown). Treatment of the β-chain with 0.5 μg V8 protease produced four major peptides with M_r of 24,000, 22,000, 15,000, and 13,000. Limited proteolysis of the α^s-chain produced four major peptides with M_r of 33,000, 20,000, 17,500, and 13,000. Digestion of p168 yielded seven major peptides, which were identical to those produced by the β- and α^s-chains. Hence, both tryptic peptide and *S. aureus* V8 protease mapping suggest that p168 contains C4 β- and α-chains. Similarly, tryptic peptide and *S. aureus* V8 protease analyses indicate that p125 contains both the α- and γ-chains (data not shown).

Partial NH_2-terminal amino acid sequencing. To define the structure of p125 and p168 better, partial NH_2-terminal sequences of p125, p168, and C4 α^s- and β-chains were obtained and compared with each other as well as with previously reported sequences (29, 30). HepG2 cells were biosynthetically labeled with [^{3}H]valine, [^{3}H]leucine, or [^{3}H]lysine, and proteins purified from SDS-polyacrylamide gels of C4 immunoprecipitates of hepatocyte media as described in Methods. 4 of the first 7 residues of p168 and the C4 β-chain and 3 of the first 11 residues of p125 (Fig. 9) and the C4 α^s-chain were determined. A comparison of the NH_2-terminal sequences and the reported sequences (29, 30) indicates that the β-chain is at the

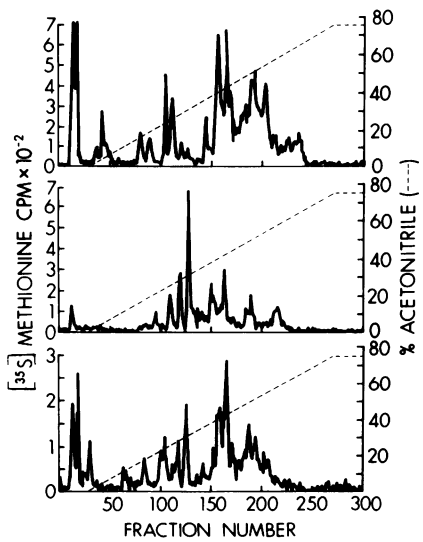

FIGURE 8 Tryptic peptide analysis of α^s- and β-chains and p168 fragment. Tryptic peptide maps of [^{35}S]methionine labeled C4α^s (*top*), C4β (*middle*), and p168 (*bottom*) were obtained by reversed-phase HPLC as described in the text. Similar tryptic peptide maps were obtained on at least two trials on two different samples.

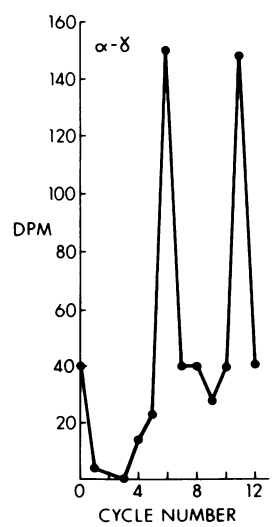

FIGURE 9 Amino acid sequence of [^{3}H]lysine-labeled p125. The radioactivity released in the butyl chloride extract at each sequencer step is presented in this figure and is representative of the results summarized in Fig. 10.

NH_2-terminus of p168 and the α-chain is at the NH_2-terminus of p125 (Fig. 10). Together with the tryptic peptide and *S. aureus* V8 protease analyses, these data confirm that p168 and p125 represent incompletely processed β-α- and α-γ-fragments of pro-C4, respectively.

A comparison of the NH_2-terminal sequences of p125 and p168 and those reported for α_2M (31) and the α- and β-chains of C3 (32, 33) confirms our initial demonstration that p125 and p168 are not related to α_2M or C3. No sequence homology was demonstrated between p168 and α_2M and between p125 and the C3 α-chain.

Pro-C4 (identified on the basis of molecular weight and immunoprecipitation by anti-C4 antisera), C4 α^s-, and C4 β-chains were evaluated in a similar fashion. The NH_2-terminal sequences of the α^s- and β-chains secreted by the hepatocytes corresponded to previously reported sequences of C4 α^p- and β-chains (Fig. 10). This is further proof that α^s (p98) is an α-chain molecule with an additional peptide with an M_r of ~5,000 at the COOH-terminus (8). The amino acid sequence of pro-C4 indicates that the β-chain is NH_2-terminal in the human C4 precursor. This finding is consistent with the β-α-γ (NH_2- to COOH-terminal) sequence determined for mouse and guinea pig pro-C4 (5, 7).

HepG2 cells secrete Chido and Rodgers variants of C4. Human C4 has been demonstrated to be encoded for by two genes (C4A and C4B) that are closely linked to the HLA-B locus (34, 35). By modifying the bis/acrylamide ratio in SDS-polyacrylamide gels, the α-chain of C4 encoded by C4A (Rodgers) has a slightly slower relative mobility than the α-chain of C4 encoded by C4B (Chido) (15). To evaluate the possibility that one peptide (β-α or α-γ) was derived from C4A (Rodgers) and the other peptide derived from C4B (Chido), labeled hepatocyte medium was immunoadsorbed with anti-Chido or anti-Rodgers alloantisera. These molecules were then compared with C4 purified by anti-C4 antisera with the modified bis/acrylamide ratio-polyacrylamide gels. Fig. 11 demonstrates that all four molecules (pro-C4, C4 β-α, C4 α-γ, and C4^s) purified by anti-C4 antisera (lane 2) were also purified by both anti-Chido (lane 3) and anti-Rodgers (lane 4) alloantisera. Hence, both Chido and Rodgers variants exist for pro-C4, C4 β-α, C4 α-γ, and C4^s secreted by the HepG2 cells. Finally, the fact that all four molecules could be purified by these alloantisera further substantiates that they are C4 molecules.

	1	5	10	15	20
α^p-chain (Ref. 29)	Asn-Val-Asn-Phe-Gln-Lys- X -Ile-Asn-Glu-Lys-Leu- X - X -Tyr-				
α^p-chain (Ref. 30)	Asn-Val-Asn-Phe-Glu-Lys-Ala-Ile-Asn-Glu-Lys-Leu-Gly-Glu-Tyr-Ala-Ser-Pro-Thr-Ala-				
p125 (α-γ)	-Val-	-Lys-	-Lys-		
α^s-chain (HepG2)	-Val-	-Lys-	-Lys-		

	1	5
β-chain (Ref. 29)	Lys-Pro-Arg-Leu-Leu-Leu-Phe- X -Pro-	
β-chain (Ref. 30)	Lys-Pro-Gly-Leu-Leu-Leu-$\frac{Phe}{Leu}$-$\frac{X}{Phe}$-Cys-	
p168 (β-α)		-Leu-Leu-Leu-
β-chain (HepG2)		-Leu-Leu-Leu-
Pro-C4 (HepG2)		-Leu-Leu-Leu-

FIGURE 10 Comparison of partial amino-terminal amino acid sequences of C4α^s, C4β, p168, p125, and pro-C4 with known sequences of α- and β-chains of C4 (23, 24), α_2M (28), and the α- and β-chains of C3 (29, 30). [^{3}H]Leucine-labeled pro-C4, p168, and C4β; [^{3}H]lysine-labeled α^s and p125; and [^{3}H]valine-labeled α^s and p125 were purified and subjected to automated microsequencing as described in Methods. Pro-C4 and p168 were sequenced two additional times with identical results. Determination of amino acid position was made independent of this laboratory.

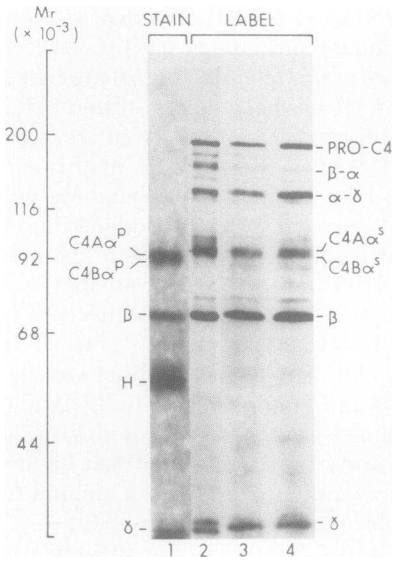

FIGURE 11 Secretion of Chido and Rodgers variants by HepG2 cells. [^{35}S]Methionine-labeled hepatocyte medium was immunoadsorbed with anti-C4 antisera (lane *2*), anti-Chido antisera (lane *3*), or anti-Rodgers antisera (lane *4*) and analyzed on a 10% polyacrylamide gel with an acrylamide/bis ratio of 1:0.006 as described in Methods. As reported by Roos et al. (15), the α^P-chain of plasma C4 from an individual heterozygous for C4A and C4B migrate as a doublet with a M_r difference of ~2,000 (lane *1*). This distinct resolution of the Chido and Rodgers C4 α-chains, however, was lost upon drying the gel for analysis by fluorography. Instead, the α^P-chain doublet became a broad band ranging from 90,000 to 94,000 D. However, the α^S-chain of C4 purified with anti-Rodgers antisera (lane *4*) appears to migrate with a M_r slightly greater than the α^S-chain of C4 purified with anti-Chido antisera (lane *3*). Furthermore, the β-α-molecule in some gels migrates as a doublet (lane *2*) and does not appear to be immunoprecipitated by the alloantisera as well as the α-γ-molecule.

DISCUSSION

In this report, we identify and structurally characterize two previously unrecognized human C4 molecules. These two molecules account for ~5% of plasma C4, incorporate methylamine, and are not related to C3 or α_2M. Furthermore, their M_r on reduced gels are appropriate for two-chain C4 molecules with uncleaved β-α- and α-γ-subunits. Structural analysis by tryptic peptide and *S. aureus* V8 protease mapping of these molecules is consistent with the hypothesis that p168 contains both the β- and α-chains and p125 contains both the α- and γ-chains. Partial NH_2-terminal sequencing demonstrates that in p168 the β-chain is NH_2-terminal and in p125 the α-chain is NH_2-terminal. Because β-α- and α-γ-fragments are not present on nonreduced gels, the remaining γ- and β-chains are presumably bound by disulfide bonds to their respective fragments.

Processing of pro-C4 to C4^s requires at least two intracellular proteolytic cleavages. Failure of either one of these cleavages will yield incompletely processed pro-C4 molecules. That both β-α- and α-γ-fragments concurrently appear extracellularly suggests that β-α cleavage is not a requirement for α-γ cleavage, and vice versa. If intracellular processing of pro-C4 to C4^s was an obligatory sequential event (e.g., β-α cleavage followed by α-γ cleavage leading to secretion), one would expect to find at least one of the two incompletely processed fragments intracellularly. Goldberger and Colten (7) have demonstrated in vitro cleavage of single chain pro-C4 from guinea pig peritoneal macrophage lysates to the three-subunit C4 molecules by plasmin. A 130,000-D molecule, consistent in size with an uncleaved α-γ-fragment, was also generated. The authors suggest that cleavage of the two intrachain sites of pro-C4 occurs in a sequential manner. In our experiments, the failure to detect either fragment intracellularly argues against such a hypothesis. Rather, both β-α and α-γ cleavages are likely to be independent of one another. These experiments, however, do not definitively address the normal sequence of the cleavages.

The lack of these fragments or C4^s in the intracellular compartment further suggests that processing of pro-C4 to C4^s is closely coupled to secretion. Roos et al. (36) have shown that glycosylation of mouse pro-C4 is important for its secretion. That β-α- and α-γ-fragments are also glycosylated is consistent with this tight linkage between secretion and glycosylation. Furthermore, these data point out that the two intracellular proteolytic cleavages are not a prerequisite for glycosylation and that glycosylation precedes proteolytic cleavage.

Aside from glycosylation and β-α and α-γ proteolytic cleavages, intracellular processing of pro-C4 also includes the formation of a thioester bond. Since both β-α- and α-γ-fragments covalently incorporate methylamine, an intact thioester bond is present in both molecules. Thus, formation of the internal thioester is independent of the two intracellular cleavages.

Although the β-α- and α-γ-fragments make up ~35% of C4 synthesized by HepG2, the two fragments account for only ~5% of plasma C4. One explanation for this discrepancy is that these incompletely processed forms have a reduced half-life in vivo. An alternative explanation is that HepG2 may not be representative of normal liver cells and inefficiently processes pro-C4 molecules. Although this possibility cannot be totally excluded, the fact that in vitro cultures of normal mouse hepatocytes and mouse peritoneal macrophages also produce large quantities of partially processed precursor molecules (37, 38) is not supportive of the latter explanation. Moreover, the concentrations in plasma appear to be less than would

be expected based on the synthesis in mouse hepatocyte cultures (P. Rosa, personal communication). Hence the discrepancies between the amounts of β-α- and α-γ-fragments from hepatoma-derived cell cultures and plasma may be accounted for by a more rapid turnover rate in vivo. However, we cannot rule out the possibility that the two fragments are further processed in plasma to the individual chains linked by disulfide bonds.

Pro-C4 is also found extracellularly in hepatocyte culture at a concentration much higher (11.5±5.4%, mean±SD) than in plasma (1–3%) (39, 40). Extracellular pro-C4 may result from inefficient intracellular cleavages, allowing pro-C4 to be secreted as a single polypeptide chain. Mouse and guinea pig pro-C4 have been shown to possess an NH_2- to COOH-terminal sequence of β-α-γ (5, 7). No sequence data, however, have been previously reported for human pro-C4. Our partial NH_2-terminal sequence revealed that the β-chain was NH_2-terminal and provides further evidence that the sequence of human pro-C4, like guinea pig and mouse pro-C4, is also β-α-γ.

Karp et al. (20) have recently observed α-γ- and presumed β-α-fragments of C4 and sex-limited protein (Slp) among various intra-H-2 recombinant strains (37). Both fragments were tightly linked to *C4* and *Slp* genes (S-region). The *cis*-dominant expression of these two incompletely processed pro-C4 molecules in F_1 mice suggested that the defect is in the structural C4 molecule, rather than in a S-region-linked protease. Based on these results, we favor the hypothesis that the human β-α- and α-γ-fragments described herein are not the result of deficiencies of intracellular proteases.

Variation in quantity among inbred strains of the α-γ- and presumed β-α-molecules was shown to be S-region controlled in the mouse (20, 37). There were also differences in the quantity of β-α and α-γ among normal human donors. Most individuals had more p168 than p125 (Fig. 1), although some had predominantly, if not entirely, p125 (Fig. 2). As in the mouse, these differences may reflect structural variations among human C4 alleles. In this regard, correlations between the quantity of β-α or α-γ in human plasma and C4 phenotype as determined by agarose gel electrophoresis could be informative.

C4 polymorphism has been described and postulated to be closely linked to the HLA-B locus (34, 35). By a modified technique of immunofixation electrophoresis, this polymorphism has been shown to be controlled by two closely linked loci, C4A (fast) and C4B (slow) (34). Awdeh and Alpers (41), using agarose gel electrophoresis, further separated six structural variants and a deletion allele at the C4A locus and two structural variants and a deletion allele at the C4B locus. Furthermore, Roos et al. (15) have visualized the Chido and Rodgers C4 α-chain variants by electrophoresis on SDS-polyacrylamide gels with a modified bis/acrylamide ratio (15). We considered the hypothesis that if one of the two fragments (β-α or α-γ) was derived from one of the two C4 genes and the other fragment derived primarily from the other gene, different β-α- and α-γ-cleavage sites may then give rise to inefficient cleavages in the respective molecules. That $C4^P$, $C4^S$, $C4_{\beta\text{-}\alpha+\gamma}$, $C4_{\beta+\alpha\text{-}\gamma}$, and pro-C4 are immunoprecipitated by both anti-Rodgers and anti-Chido alloantisera rules out this hypothesis.

In summary, C4 appears to be composed of a family of structurally distinct molecules: $C4^P$, $C4^S$, $C4_{\beta\text{-}\alpha+\gamma}$, $C4_{\beta+\alpha\text{-}\gamma}$, and pro-C4 are all found in plasma. Since all five molecules are found for both C4A and C4B genes by SDS-PAGE, at least 10 structurally distinct forms of C4 can be found in human plasma (Table I). Although some of these molecules may be of limited physiologic importance, they provide important information concerning intracellular and extracellular processing (Fig. 12). After translation of C4 messenger RNA, C4 precursor is processed further by formation of the appropriate disulfide bonds (1), glycosylation of the α^S-chain with a complex sugar and the β-chain with a high mannose sugar (29, 30), and formation of the internal thioester bond (42). Intracellular proteolytic cleavage of the β-α and α-γ bonds (Fig. 12, A) yields the secreted molecule $C4^S$. Upon secretion, the presumed extracellu-

TABLE I
Definition of Five Structurally Distinct C4 Molecules

C4 molecule	Structure* (NH_2- to COOH-terminal sequence)	Percentage of total C4 secreted by HepG2 cells‡	Percentage of total C4 found in EDTA-plasma§
pro-C4	$\beta - \alpha^S - \gamma$	11	1–3‖
p168	$\beta - \alpha^S + \gamma$	14	~3
p125	$\beta + \alpha^S - \gamma$	21	~1
$C4^S$	$\beta + \alpha^S + \gamma$	55	8
$C4^P$	$\beta + \alpha^P + \gamma$	<1¶	85

* (+) denotes covalently linked chains via disulfide bonds that can be reduced to the appropriate subunits while (−) denotes uncleaved molecule.

‡ These percentages represent the mean of five experiments as determined by densitometric scanning.

§ With the exception of pro-C4, these percentages were quantitated by densitometric scanning of [^{14}C]methylamine-labeled C4 from EDTA-plasma.

‖ See references 39 and 40.

¶ A molecule with a slightly lower molecular weight than the plasma α^P-chain is secreted by the HepG2 cells. This molecule makes up <1% of the total secreted C4 and may represent an incompletely glycosylated variant of C4 (8).

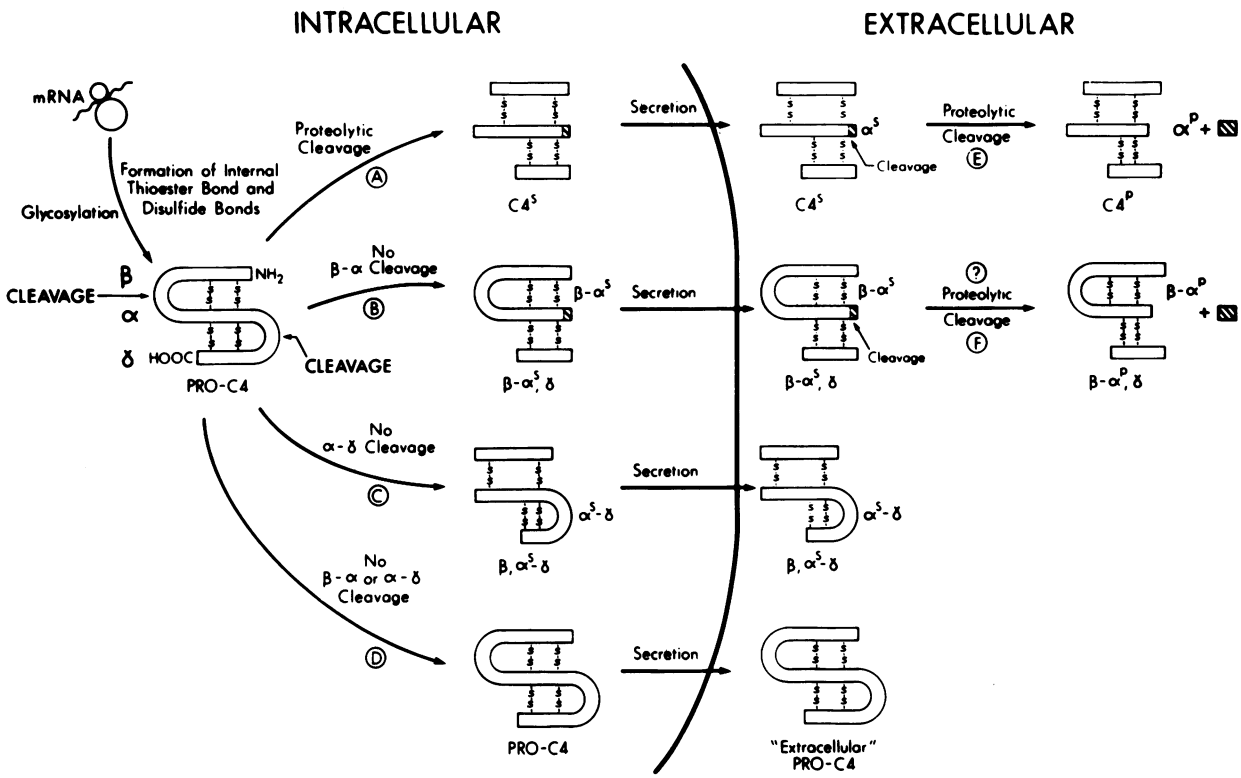

FIGURE 12 Model of C4 biosynthesis and processing. See text for discussion.

lar proteolytic cleavage (*E*) of an apparent ~5,000-D COOH-terminal fragment of the α^s-chain results in the predominant form of C4 found in plasma, $C4^P$ (8, 9). If either β-α (*B*) or α-γ (*C*) cleavages do not occur, two chain subunits with uncleaved β-α or α-γ subunits of pro-C4, respectively, are produced. These molecules, in turn, are secreted as incompletely processed fragments of pro-C4. The β + α-γ-molecule presumably cannot undergo further extracellular processing, but the β-α + γ-molecule with an exposed α^s-chain COOH-terminus may undergo extracellular processing (*F*) to yield α^P. Similarly, if neither β-α nor α-γ cleavages occur, pro-C4 may be secreted and may circulate as a single-chain polypeptide. It is likely that other multichain proteins derived by proteolytic processing of a single chain precursor will demonstrate a similar array of molecules.

ACKNOWLEDGMENTS

We thank Lorraine Whitely for typing the manuscript, Harold Sims for technical assistance, and Drs. David Karp, Theodore Munns, Patricia Rosa, Arnold Strauss, and Donald Shreffler for review and discussion of this manuscript.

This work was supported by the Howard Hughes Medical Institute and National Institutes of Health training grant GM-07200 (Medical Scientist Award to Dr. A. C. Chan).

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