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Immunology

Characterization of the M_r difference between secreted murine fourth component of complement and the major plasma form: Evidence for carboxyl-terminal cleavage of the α chain

(complement/proteolytic processing)

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ABSTRACT **The α-chain of murine fourth component of complement (C4) secreted by cells *in vitro* and *in vivo* has a M_r that is larger by ≈4,000 than that of the α-chain of the principal form of C4 in plasma. By using *in vivo* labeling of C4 with [^{35}S]methionine, C4 was shown to be first synthesized with the higher M_r ("secreted") α-chain, which was then quickly processed ($t_{1/2} \approx 1$ hr) extracellularly to the mature ("plasma") C4 possessing the lower M_r α-chain. Both forms of C4 were functional as assayed by the ability of their α-chains to be cleaved by the protease $\overline{C1}$, to bind methylamine, and to undergo denaturation-dependent autolysis. When secreted C4 and plasma C4 were activated to C4b, the M_r difference of 4,000 was maintained in the α′-chains. The M_r difference was localized to the carboxyl-terminal autolytic fragment of the α-chain and was unaffected by the removal of carbohydrate. C4 from resident peritoneal macrophage cultures could be converted to the plasma form by incubation with heparin/plasma. This conversion could be blocked by EDTA or 1,10-phenanthroline. These data suggest that an enzyme, presumably a neutral proteinase present in mouse plasma, cleaves the carboxyl terminus of newly synthesized C4 α-chains, thereby creating the major form of C4 in plasma.**

The fourth component of murine complement (C4) is a plasma glycoprotein with a M_r of ≈200,000. The native molecule contains three disulfide-linked polypeptides, termed α, β, and γ, which are derived from a single-chain intracellular precursor (1–4). The structural gene for murine C4 has been mapped to the *S* region of *H-2*, the murine major histocompatibility complex (1, 5, 6).

In 1978, Roos *et al.* (1) demonstrated the biosynthesis of C4 by resident murine peritoneal macrophages. At that time, they described two types of variation in the mass of the C4 α-chain. Most mouse strains used had a C4 α-chain with a M_r of ≈98,000, whereas strains bearing the S^{w7} haplotype had α-chains with a M_r of ≈94,000. This variation has recently been shown to be due to differences in the degree of glycosylation of the α-chain (7). Secondly, when the biosynthetically radiolabeled α-chain from macrophage culture medium was compared to the α-chain found associated with C4 in plasma, it was found that the plasma α-chain M_r was ≈4,000 less than that of the culture-derived form. This was true in all seven mouse strains tested. In contrast, the β-chains (M_r, ≈74,000) and γ-chains (M_r, ≈34,000) of culture-derived C4 were identical to their plasma counterparts.

We now report that C4 containing the higher M_r form of the α-chain (α^s-chain) is the primary product synthesized by C4-secreting cells *in vivo* and is rapidly processed by specific proteolysis to the plasma form of C4 containing the lower M_r form of the α-chain (α^p-chain).‡ This proteolytic cleavage can be reproduced *in vitro*, thus potentially allowing characterization of the proteinase, the cleaved C4 α-chain, and the cleavage fragment.

Portions of this work were presented earlier in preliminary form (9).

MATERIALS AND METHODS

Animals. All mice used in this study were produced in the colony of the Department of Genetics, Washington University School of Medicine. Strains carrying the $C4^{w7}$ gene (B10.WR and C3H.WS1p) were used to obtain C4 having a M_r 94,000 α-chain in culture; strains with the $C4^d$ gene [B10.A, B10.A(1R), and B10.A(2R)] were used to obtain C4 having the M_r 98,000 α-chain (1).

Cultures and Immunoprecipitation. C4 was biosynthetically radiolabeled with [^{35}S]methionine (Amersham) in cultures of resident peritoneal macrophages as described (7, 8). It was purified from culture medium by direct immunoprecipitation with isologous plasma as carrier (1). Precipitates were analyzed by reducing or nonreducing $NaDodSO_4$/polyacrylamide gel electrophoresis as described by Laemmli (10).

Hepatocyte Cultures. Parenchymal hepatocytes were obtained from adult male mice by the procedure of Renton *et al.* (11). Cells (6×10^6) in RPMI 1640 medium containing 10% fetal bovine serum, glutamine, and antibiotics were plated on collagen gels (12) in 25-cm^2 tissue culture flasks. After 1 hr at 37°C, the nonadherent cells were removed, and the medium was replaced with methionine-free RPMI 1640 medium (GIBCO), supplemented as above and containing 300 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine. Labeling continued for 20–24 hr, at which time C4 protein was isolated from the culture medium as above.

***In Vivo* Labeling.** [^{35}S]Methionine (5 mCi) as supplied by the manufacturer was lyophilized and resuspended in 0.25 ml of Hanks' balanced salt solution and injected intraperitoneally into an adult male mouse. The mouse was bled from the retro-orbital sinus at the times indicated, and the blood immediately was

Abbreviations: C1, C2, C3, C4, first, second, third, and fourth components of complement; α^s-chain, α-chain of C4 secreted by peritoneal macrophages or hepatocytes; α^p-chain, α-chain of the major form of C4 isolated from plasma; pro-C4, precursor to C4.

‡ C4 protein exists in plasma in one major and several minor forms. The major form has the lower M_r α-chain, disulfide-bonded to the β- and γ-chains. The minor forms (≈20%) include (*i*) C4 with an α-chain having a higher M_r that matches the α^s-chain, (*ii*) the precursor to C4 (pro-C4) having a single polypeptide chain, and (*iii*) partially processed pro-C4s having a two-polypeptide structure (8). In this communication, the term "plasma form" of C4 will refer to the major form seen in polyacrylamide gel analyses.

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made 10 mM in EDTA and 2 mM in phenylmethylsulfonyl fluoride. These EDTA/plasma samples were stored at −80°C until used for immunoprecipitation.

Removal of Carbohydrate from C4. C4 was deglycosylated by using a modification of the method of Edge *et al.* (13) as described (7). Basically, immunoprecipitates were incubated at 0°C in trifluoromethanesulfonic acid/anisole, 9:1 (vol/vol), for 2 hr, the mixture was neutralized with 10% pyridine in cold (−40°C) diethyl ether, and the resultant precipitate was dialyzed against 0.1 M ammonium bicarbonate prior to electrophoresis.

Labeling at the Internal Thioester and Autolytic Cleavage. The α-chain of murine C4 contains a methylamine-reactive internal thioester bond (8). Immunoprecipitates were suspended in [^{14}C]methylamine (200 μCi/ml; New England Nuclear) in 0.1 M Tris acetate (pH 8.5) for 4 hr at 37°C. Precipitates were then washed three times with 10 mM EDTA (pH 7.4) and electrophoresed. The presence of an intact thioester in [^{35}S]methionine-labeled C4 was determined by heating these immunoprecipitates in 0.1% $NaDodSO_4$/0.1 M Tris acetate, pH 8.5, at 75°C for 30 min. Under these conditions, the thioester rearranges, causing a single peptide bond cleavage in the α-chain. Concentrated electrophoresis sample buffer containing 2-mercaptoethanol was then added to the denatured precipitates.

Fluid-Phase Activation of C4 to C4b. Blood was collected and allowed to clot on ice in the presence of K76 monocarboxylic acid (2.5 mg/ml), a fungal inhibitor of the C4b inactivator, (courtesy of Wasei Miyazaki, Otsuka Pharmaceutical, Tokushima, Japan). Fresh serum (50 μl) was mixed with 250 μl of [^{35}S]methionine-labeled macrophage culture medium (6-hr incubation), after which soybean trypsin inhibitor (Sigma) was added to 1 mg/ml, and the K76 monocarboxylic acid concentration was adjusted to 2.5 mg/ml. Activation of C4 was accomplished by addition of 150 μg of heat-aggregated (60°C for 20 min) bovine gamma globulins and incubation at 37°C for 1 hr. Control samples were made 10 mM in EDTA prior to incubation. After incubation, the aggregated gamma globulins were removed by centrifugation, and the C4 was immunoprecipitated.

Heparin Plasma Treatment of Secreted C4. Macrophage culture medium containing radiolabeled C4 was mixed 1:1 (vol/vol) with isologous plasma containing 10 units of heparin (Abbott; preservative free) per ml and incubated for various times at 37°C. The incubation was stopped by the addition of cold EDTA (final concentration, 10 mM), and the C4 was immunoprecipitated by using the heparin/plasma as carrier. In some cases various protease inhibitors were present during the incubation.

RESULTS

Hepatocyte and Macrophage-Derived C4 Molecules Have the Same M_r α-Chain. The liver is the major site of C4 synthesis in the mouse (14). The difference in α-chain M_r may then reflect tissue-specific gene expression, as has been proposed for other complement proteins (15). Biosynthetically radiolabeled C4 from hepatocyte and macrophage cultures was compared to C4 from plasma in which the α-chain had been labeled with [^{14}C]methylamine (Fig. 1). The M_r of the α^s-chain from hepatocytes was the same as that from macrophages and was ≈4,000 larger than that of the α^p-chain. This M_r difference was also seen in intact C4 electrophoresed under nonreducing conditions (not shown). Careful inspection of the original autoradiograph revealed methylamine label in both the α^p-chain and the small amount of α^s-chain present in plasma (see *Discussion*). The amount of label was proportional to the amount of protein present as judged by Coomassie brilliant blue staining. In this instance, S^{w7} haplotype mice were used. Therefore the M_rs of the two α-chains are ≈94,000 and ≈90,000. This experiment was repeated with S^f and S^d haplotype mice with identical results except that the M_rs of the two α-chains of these haplotypes were ≈98,000 and ≈94,000, due to the presence of additional carbohydrate (7).

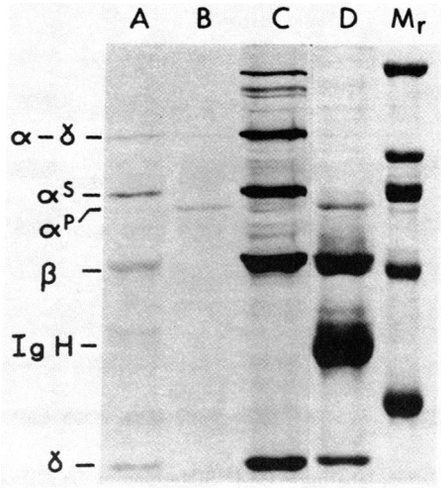

FIG. 1. Comparison of hepatocyte-, macrophage-, and plasma-derived C4 α-chains. Hepatocytes and peritoneal macrophages from mice bearing the S^{w7} haplotype were cultured in the presence of [^{35}S]methionine, and the radiolabeled C4 was immunoprecipitated from the culture medium. The α-chain of murine plasma C4 was labeled at the internal thioester by incubating C4-containing immunoprecipitates with [^{14}C]methylamine. Immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol, and the gels were stained, dried, and autoradiographed. Lanes: A, [^{35}S]methionine-labeled C4 from primary hepatocyte culture; B, [^{14}C]methylamine-labeled C4 from plasma; C, [^{35}S]methionine-labeled C4 from peritoneal macrophage culture; D, Coomassie brilliant blue-stained gel from which the autoradiograph in lane B was made. Standard proteins and their M_r values are: myosin (200,000), β-galatosidase (116,500), phosphorylase b (94,000), bovine serum albumin (68,000), and ovalbumin (43,000). The higher M_r bands (>120,000) are pro-C4 and several incompletely processed fragments of pro-C4 (8).

The C4 α-Chain Is Rapidly Processed *in Vivo*. To rule out the possibility that the α^s-chain is the product of a gene expressed only by cells in culture or that the processing of C4 *in vitro* is defective, *de novo* synthesized C4 was radiolabeled with [^{35}S]methionine *in vivo*. Bleedings were performed at 1, 2, 4, 8, 12, and 24 hr after injection of 5 mCi of labeled amino acid. The radiolabeled C4 protein isolated from these bleedings is shown in Fig. 2. At 1 hr, newly synthesized C4 was barely apparent, consistent with the kinetics of C4 synthesis (refs. 2 and 4; unpublished observations). Most of the α-chain was in the higher M_r form, identical to the α-chains isolated from macrophage cultures (Fig. 2, lanes C). At 2 hr, ≈60% of the radiolabeled C4 was α^s-chain and the other 40% was α^p-chain. The labeled α-chain was "chased" into the lower M_r form over the next 6–10 hr. At 24 hr, much of the radiolabeled C4 remained in the plasma, all of it having the α^p-chain. Therefore, the α^s-chain not only is found in C4 derived from cultured cells but also is a component of *in vivo* synthesized C4. This α-chain is rapidly processed to the lower M_r α^p-chain associated with the major form of C4 in plasma.

Characterization of the M_r Difference Between the α^s-Chain and the α^p-Chain. C4, like C3 (third component of complement) and α_2-macroglobulin, contains an internal thioester moiety that may form the covalent binding site between it and biologic materials (16–21). When polypeptides containing the

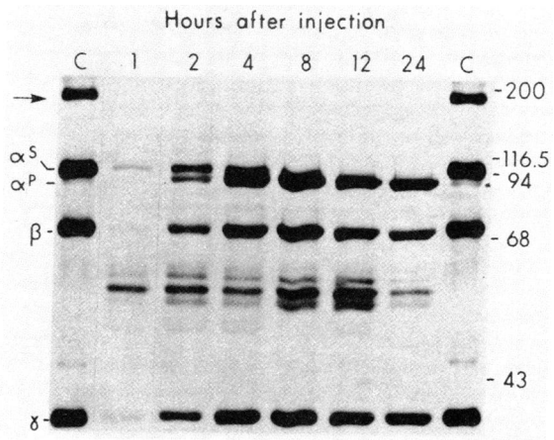

FIG. 2. *In vivo* labeling of C4. [^{35}S]Methionine (5 mCi) was injected intraperitoneally into an S^d-haplotype mouse. Bleedings (≈0.2 ml) were taken at 1, 2, 4, 8, 12. and 24 hr, and the blood was made 10 mM in EDTA and 2 mM in phenylmethylsulfonyl fluoride. C4 was immunoprecipitated from these plasma samples and electrophoresed; lanes C contain C4 labeled with [^{35}S]methionine in peritoneal macrophage culture. The unidentified series of bands between the C4 β- and γ-chains are probably nonspecifically precipitated immunoglobulin heavy chains. The band indicated by the arrow is presumably an incompletely processed fragment of pro-C4.

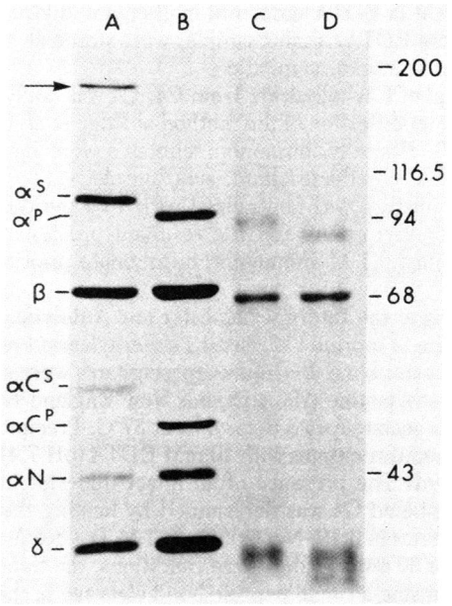

FIG. 3. Characterization of plasma-derived C4 α-chains. C4 was radiolabeled with [^{35}S]methionine, either in peritoneal macrophage culture (lanes A and C) or after 24-hr *in vivo* labeling (lanes B and D) (see Fig. 2). One set of C4 immunoprecipitates was heated in 0.1 M Tris acetate, pH 8.5/0.1% NaDodSO$_4$ at 75°C for 30 min to effect autolytic cleavage of the α-chains. Another set of precipitates was deglycosylated with trifluoromethanesulfonic acid. Lanes: A, autolyzed C4 from macrophage culture; B, autolyzed C4 from plasma; C, deglycosylated C4 from macrophage culture; D, deglycosylated C4 from plasma. αN is the amino-terminal autolytic fragment of the α-chain (M_r, ≈40,000); αC^P is the carboxyl-terminal fragment from plasma C4 (M_r, ≈54,000); αC^s is the carboxyl-terminal fragment from secreted C4 (M_r, ≈58,000). The shift in β-chain mobility is due to the loss of high mannose type oligosaccharide (7). The broadening of the γ-chains is not characteristic (7). The band indicated by the arrow is presumably an incompletely processed fragment of pro-C4.

intact thioester are denatured, they autolyze at the peptide bond carboxyl-terminal to the glutamic acid involved in ester formation (21–25). Presumably this is due to an internal rearrangement forming a pyroglutamic acid residue (25, 26). In Fig. 3, lanes A and B show C4 from a macrophage culture and *in vivo* radiolabeled plasma (24 hr after synthesis; see Fig. 2), respectively. They were heated at 75°C for 30 min in 0.1% NaDodSO$_4$ to effect autolytic cleavage of the α-chains. The α^s-chain produced M_r ≈40,000 and ≈58,000 fragments; the α^P-chain produced M_r ≈40,000 and ≈54,000 fragments. Autolysis did not go to completion, presumably due to hydrolysis of the thioester prior to or during the denaturation step. Studies of human C4 (21) and our own work (unpublished data) have shown that the smaller fragment is amino-terminal in the intact α-chain. Therefore, the M_r difference between culture- and plasma-derived α-chains is localized to the carboxyl-terminal portion of the subunit.

Differential glycosylation of the carboxyl-terminal autolytic fragment of the α-chain results in allelic variation of murine C4 (7). To investigate this as the cause of M_r variation between culture- and plasma-derived α-chains, immunoprecipitates were treated with trifluoromethanesulfonic acid to remove all *N*- and *O*-linked carbohydrate (13). In Fig. 3, lanes C and D show deglycosylated C4 from macrophage culture and plasma. The M_r 4,000 difference was maintained after removal of carbohydrate. Thus, the difference between the α^s-chain and the α^P-chain appears to be in the protein portion of the molecule.

Both Forms of C4 α-Chain Are Cleaved by the First Component of Complement ($C\bar{1}$). Aggregated immunoglobulin is capable of activating the classical pathway of complement *in vitro* (27). When aggregated bovine gamma globulin was added to a mixture of murine serum and radiolabeled macrophage culture medium, two distinct α′-chains appeared (Fig. 4). The M_r of the α′-chain visible in the Coomassie brilliant blue-stained gel was ≈4,000 lower than that of the major α′-chain seen in the corresponding autoradiograph. A minor, radiolabeled α′-chain was present that corresponded to the stained protein band. Presumably, this represents activation of the small amount of radiolabeled C4 that was already in the mature, plasma form (see *Discussion*). These data confirm that fact that the M_r difference is *not* at the amino terminus and that both forms of C4 are capable of being activated by $C\bar{1}$. The latter point also has been inferred from the fact that mouse plasma, macrophage cultures, and hepatocyte cultures contain hemolytically active C4 (refs. 28 and 29; unpublished data).

The α^s-Chain Can Be Converted to the α^P-Chain by Incubation with Heparin/Plasma. In a preliminary effort to locate the factor(s) responsible for conversion of the α^s-chain to the lower M_r form, macrophage culture medium was incubated with isologous mouse serum or plasma. The effect of heparin/plasma on macrophage-derived C4 is seen in Fig. 5. At zero time (lane A), the α^s-chain predominated. (A variable amount of a lower M_r α-chain was present in all preparations of macrophage- and hepatocyte-derived C4 *in vitro*.) As incubation at 37°C progressed, the amount of α^s-chain diminished and the α^P-chain accumulated. No effect was seen on the β- or γ-chains. After 1 hr, ≥50% of the original α-chain was converted. Further incubation (up to 24 hr) resulted in only a modest increase in α^P-chain. Incubation of macrophage-derived C4 with heparin alone (10 units/ml) (Fig. 5, lane G) did not cause conversion; neither did incubation with heparin/plasma at 4°C or incubation with heat-inactivated (56°C for 60 min) plasma (not shown). Lanes I and J show culture-derived C4 before and after a 1-hr incubation with heparin/plasma, which was then denatured to cause autolytic cleavage. In the 1-hr sample (lane J), both forms of α-chain were observed to autolyze, indicating that conversion to the plasma form does not destroy the internal thioester.

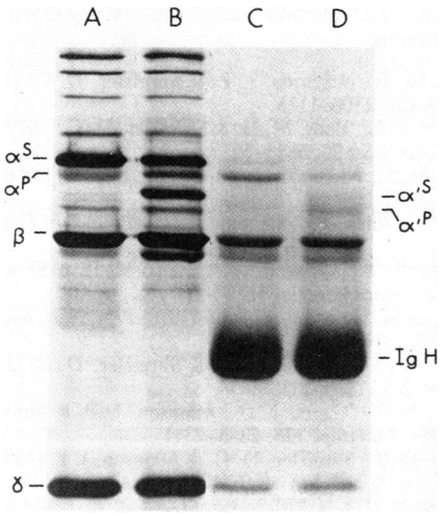

FIG. 4. Fluid-phase activation of C4. Mouse serum and [^{35}S]methionine-labeled culture medium were mixed in the presence of the protease inhibitors, soybean trypsin inhibitor (1 mg/ml), and K76 COONa (2.5 mg/ml). Heat-aggregated bovine gamma globulin (0.5 mg/ml) was added, and the mixture was incubated with or without 10 mM EDTA at 37°C for 1 hr. The C4 was then immunoprecipitated and analyzed by electrophoresis under reducing conditions. Lanes: A and C, C4 incubated in the presence of 10 mM EDTA; lanes B and D, C4 activated in the absence of EDTA. Lanes A and B constitute an autoradiograph of the gel containing lanes C and D (Coomassie brilliant blue stain). In lane A, a small amount of α'-chain can be seen, along with several higher M_r bands, which are pro-C4 and incompletely processed fragments of pro-C4. In lane B, the amount of α'-chain is increased after activation of C4 to C4b. There is also an increase in the amount of a protein migrating just ahead of the β-chain, which is probably an α'-chain-derived fragment. In lane C, the protein stain demonstrates the two forms of α-chain present in plasma C4 prior to activation. After activation by $C\bar{1}$ (lane D), the levels of stained α-chains diminish, and the α'-chains of plasma C4 are visible.

Again, it was the carboxyl-terminal portion of the α-chain that was processsed, producing fragments similar if not identical to those obtained from authentic *in vivo* synthesized C4 (see Fig. 3).

Lane H in Fig. 5 contains culture-derived C4 that had been treated with heparin/plasma made 10 mM in EDTA. The conversion of the α-chain was abrogated by the chelating agent. 1,10-Phenanthroline (5 mM) was also effective in preventing conversion (not shown). Protease inhibitors that were ineffective at preventing conversion (not shown) were: diisopropyl fluorophosphate (2.5 mM), phenylmethylsulfonyl fluoride (2 mM), iodoacetamide (20 mM), ε-aminocaproic acid (25 mM), benzamidine hydrochloride (5 mM), L-1-tosylamide-2-phenylethyl chloromethyl ketone (50 μg/ml), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (50 μg/ml), pepstatin (10 μM), leupeptin (15 μg/ml), aprotinin (1 unit/ml), and soybean trypsin inhibitor (1 mg/ml). These data strongly suggest that the proteinase responsible for *in vitro* conversion of the α^s-chain is a metal-dependent enzyme and not a serine or sulfhydryl proteinase.

DISCUSSION

The data shown here demonstrate that murine C4 is secreted from cells having an α-chain with a M_r that is ≈4,000 larger than that of the α-chain associated with C4 in plasma. A proteolytic cleavage takes place rapidly at the carboxyl terminus of the α-chain, reducing the M_r. The resulting C4 molecule is stable in plasma and retains functional properties. This proteolytic cleavage can be mimicked *in vitro* by incubation of biosynthetically radiolabeled C4 from cell culture with murine plasma. Based

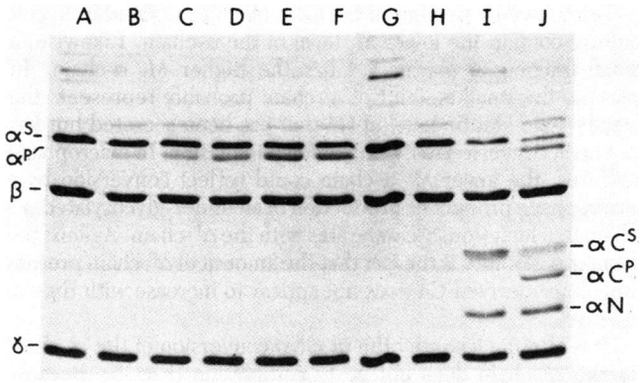

FIG. 5. Incubation of culture-derived C4 with heparin/plasma. [^{35}S]methionine-labeled macrophage culture medium was mixed 1:1 (vol/vol) with fresh isologous plasma collected in heparin (10 units/ml). The mixture was incubated at 37°C for 0 (lane A), 5 (lane B), 10 (lane C), 20 (lane D), 40 (lane E), or 60 (lane F) min before immunoprecipitation. Lanes G–J: G, culture medium incubated 1 hr at 37°C with an equal volume of phosphate-buffered saline containing 10 units of heparin per ml; H, culture medium plus heparin/plasma with 10 mM EDTA incubated 1 hr at 37°C; I and J, culture media incubated with heparin/plasma for 0 (lane I) or 60 (lane J) min. The latter two immunoprecipitates were heated in 0.1% $NaDodSO_4$ at 75°C for 30 min prior to reduction and electrophoresis to effect autolytic cleavage of the C4 α-chains. The high M_r bands (>120,000) are pro-C4 and incompletely processed fragments of pro-C4.

on inhibitor experiments, the proteinase responsible appears to be a member of the neutral, metal-dependent category (30).

The functional significance of this cleavage is not known. Both the secreted and plasma-derived forms of C4 are sensitive to cleavage by $C\bar{1}$ in the setting of classical pathway activators such as heat-aggregated gamma globulin (Fig. 4) or antibody-coated erythrocytes (unpublished data). This fact and the demonstrated levels of hemolytically active C4 in both macrophage culture medium and plasma (28, 29) suggest that the α-chain processing neither activates nor inactivates the molecule. Thus, the binding of C2 and the role of C4 in the C3 convertase are intact in the secreted form. (The interaction of the higher M_r form with the C4 binding protein, the C3b/C4b inactivator, and membrane receptors for C4b have not been studied.) This activity of the secreted form is supported by the fact that both molecules are capable of binding methylamine and of autolytic cleavage, both of which are processes requiring an intact thioester. Cleavage of the thioester eliminates C4 hemolytic activity (21, 31). The hemolytic efficiency of the two types of C4 molecules may be different. It has been shown that decreased glycosylation of the carboxyl-terminal portion of the C4 α-chain is associated with decreased hemolytic efficiency (7, 28). This question can be resolved by comparing the hemolytic efficiency of the two molecules in question. Alternatively, this cleavage may point to an as yet undefined function of C4 or merely to a functionally unimportant interaction between C4 and a nonspecific or crossreacting proteinase. The released carboxyl-terminal fragment of the α-chain may have a role in the biosynthesis of C4, such as aiding the intracellular transport of the molecule or influencing the three-dimensional conformation in order to allow formation of the internal thioester. It may have a distinct biological activity, such as that of C3a, C4a, or C5a. Finally, the M_r 4,000 fragment may serve as a signal for the regulation of C4 synthesis. This type of peptide fragment regulation has recently been shown for prothrombin (32) and guinea pig C4 (33). As levels of the C4 α-chain fragment increase, hepatic C4 synthesis would decrease, thus maintaining the steady-state level of the protein.

Ten to twenty percent of the C4 molecules isolated from cell culture contain the lower M_r form of the α-chain. Likewise, a small fraction of plasma C4 has the higher M_r α-chain. In plasma, the small amount of α^s-chain probably represents the steady-state kinetic level of C4 that has been secreted but has not been converted to the major form in plasma. In macrophage cultures, the lower M_r α-chain could reflect conversion by a macrophage product or production of an under-glycosylated α^s-chain that fortuitously comigrates with the α^p-chain. Against the former possibility is the fact that the amount of α^p-chain present in culture-derived C4 does not appear to increase with time in culture.

It is also unclear why the *in vitro* conversion of the α^s-chain to the α^p-chain does not go to completion (Fig. 5). Because α-chains that have had spontaneous hydrolysis of the thioester cannot be cleaved by the protease $\overline{C1}$ (21) and because the thioester cleavage produces conformational changes (34, 35), it was possible that, in fact, the resistant α^s-chains had lost the internal thioester. However, a large portion of these α-chains are able to undergo autolytic cleavage, indicating the presence of an intact thioester bond.

The processing of the C4 α-chain described here is the third, defined, postsynthetic proteolysis of this polypeptide. During the activation of C4, the serine protease $\overline{C1s}$ cleaves the C4a fragment comprising the first 77 amino acids from the amino terminus of the α-chain (36). After complement activation, factor I with a Ca^{2+}-dependent cofactor, C4-binding protein, cleaves the α'-chain of the C4b molecule in the fluid phase (37) and on cell surfaces. In contrast to these cleavage events, which lead to the activation of and ultimately to the degradation of the C4 molecule, the removal of the carboxyl-terminal portion of the α-chain leaves a structurally stable, fully functional molecule. No similar extracellular processing has been described for any of the other complement components. However, in most cases biosynthetically radiolabeled material has not been compared directly with plasma proteins. One protein, not in the complement system, in which this has been examined, is collagen. Procollagen is secreted from cells as a helix of three identical disulfide-bonded subunits. In the extracellular space, amino-terminal and carboxyl-terminal propeptides are cleaved from each chain in the triple helix (38). The proteases responsible for these cleavages are also neutral, metal-dependent enzymes. With careful scrutiny, it is likely that additional proteins that undergo postsecretory maturation will be found.

Sex-limited protein (Slp) is a homologue of murine C4 that lacks hemolytic activity (3, 28). It has an α-chain of $M_r \approx 105{,}000$. Most, if not all, of the M_r difference between the C4 and Slp α-chains is eliminated by removal of carbohydrate (39). The secreted form of the Slp α-chain and the plasma form of the Slp α-chain have the same M_r, and the Slp α-chain is not cleaved by plasma *in vitro* (not shown). We postulate that the extra carbohydrate interferes with the action of the α-chain-cleaving enzyme, or that the Slp α-chain lacks the cleavage site.

Finally, recent experiments suggest that human C4 is processed in a manner identical to murine C4. We have found high and low M_r forms of human C4 α-chain and have used human heparin/plasma to process murine macrophage-derived C4 (unpublished data). This should facilitate both the isolation of the enzyme responsible for α-chain cleavage and the characterization of the substrate (i.e., newly secreted C4).

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