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Characteristics of complement subcomponents C1r and C1s synthesized by Hep G2 cells

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The association and activation states of complement subcomponents C1r and C1s biosynthesized by Hep G2 cells were studied. C1r and C1s are secreted in stoichiometric amounts; in the presence of Ca^{2+} they are associated in a complex that sediments similarly to plasma $C1r_2$–$C1s_2$. Both compounds are synthesized as monomer proteins of apparent M_r 86000. C1r is secreted as a dimer. Secreted C1r is not autoactivatable but undergoes proteolysis by exogenous $C\bar{1}r$; secreted C1s is also proteolysed by exogenous $C\bar{1}r$. In the presence of immune-complex-bound C1q, secreted C1r and C1s are able to reconstitute C1, but normal activation requires extrinsic $C\bar{1}r_2$–$C\bar{1}s_2$.

INTRODUCTION

The classical pathway of complement is triggered by the activation of component C1. C1 is formed of two distinct subunits, C1q and $C1r_2$–$C1s_2$, a Ca^{2+}-dependent association of the C1r and C1s subcomponents. C1q binds to activators and transmits an activation signal to $C1r_2$–$C1s_2$, which contains the catalytic potential.

Purified human C1 subcomponents have been studied in detail in this laboratory. Dimeric C1r has been shown to play a key role in C1 activation. Isolated dimeric C1r is autoactivated on incubation at 37 °C by an inter-monomer cross-activation mechanism (Arlaud *et al.*, 1980). Autoactivation potential of $C1r_2$ is influenced by its association state. The $C1r_2$–$C1s_2$ subcomplex is stable when isolated (Colomb *et al.*, 1984). In contrast, when $C1r_2$–$C1s_2$ is associated to C1q inside C1, activation of C1 is possible, which reflects a modulation of the activatability of $C1r_2$ (Ziccardi, 1982). The enzymic activity of C1 is borne by the C1s subcomponent. Activated C1s cleaves C4 and C2, allowing the formation of the C3 convertase complex.

The purpose of the present paper is to analyse the structure–function relationships of nascent C1r and C1s, the characteristics of the purified proteins being taken as a reference. To this end we studied the activation and association states of C1r and C1s during their biosynthesis by Hep G2 cells. Most of the complement proteins, except C1q, C7 and C9, have been shown to be synthesized and secreted by this human hepatoma-derived cell line (Morris *et al.*, 1982). The absence of C1q biosynthesis may reflect the situation *in vivo*, where subcomponents of different cellular origins contribute to the integrity of C1 in serum.

MATERIALS AND METHODS

Materials

Hep G2 cells were a gift from Dr. T. Meo (Paris, France). RPMI 1640 medium, foetal-calf serum (heat-inactivated), penicillin, streptomycin and fungizone were purchased from Flow Laboratories. Selectamine was from Flobio. IgG-Sorb was purchased from the Enzyme Center. L-[^{35}S]Methionine (specific radioactivity approx. 1000 Ci/mmol) and [^{14}C]methylated M_r markers were obtained from Amersham International. En^{3}Hance was from New England Nuclear.

Proteins and antisera

$C\bar{1}r$ and $C\bar{1}s$ were purified as described by Arlaud *et al.* (1979). Antisera to heat-aggregated $C\bar{1}r$ and glutaraldehyde-insolubilized $C\bar{1}s$ were raised in rabbits (Porter 1955). Immunoglobulins were obtained by Na_2SO_4 precipitation of rabbit sera. Specific antibodies to $C\bar{1}s$ were purified by affinity chromatography (Villiers *et al.*, 1982). Anti-$C\bar{1}r$ immunoglobulins were systematically adsorbed on Sepharose-coupled human albumin.

Preparation of immune aggregates

Immune aggregates were prepared at equivalence as described by Arlaud *et al.* (1979) with hen ovalbumin and anti-ovalbumin IgG purified from rabbit antisera.

Preparation of C1-depleted serum

Human serum was depleted of C1 by adsorption on IgG–ovalbumin aggregates. Immune aggregates were suspended in the serum at the concentration of 1 mg/ml and the mixture was incubated for 40 min at 4 °C. After centrifugation, the supernatant was treated twice more in the same manner. Less than 10% residual C1 was present in the C1-depleted serum as assessed by haemolytic activity (Fischer, 1965).

Cell culture and biosynthetic labelling

Hep G2 cells were grown almost to confluence in 25 cm^2 plastic flasks (Falcon) at 37 °C in a humidified atmosphere of air/CO_2 (19:1), with RPMI 1640 medium supplemented with 20 mM-Hepes, 1 mM-pyruvate, 100

Abbreviations used: the nomenclature of the complement components and subcomponents is that recommended by the World Health Organisation (1968); activation of a component is indicated by an overbar.

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units of penicillin/ml, 100 μg of streptomycin/ml, 2.5 μg of fungizone/ml and 10% (v/v) foetal-calf serum. Before being labelled the cells were incubated for 48 h in the same medium containing 10% (v/v) of conditioned medium, which had been prepared as described by Bensa *et al.* (1983) from staphylococcal-endotoxin-A-stimulated lymphocytes. After being washed twice with Hanks balanced salt solution, cultures were incubated for 17 h with 4 ml of Selectamine lacking methionine supplemented with 10% (v/v) foetal-calf serum, 10% (v/v) conditioned medium and 250 μCi of L-[^{35}S]methionine/ml. After incubation the medium was removed, centrifuged at 1500 *g* for 10 min, dialysed against phosphate-buffered saline (0.145 M-NaCl/0.02 M-sodium phosphate buffer, pH 7.4) and stored at −20 °C. The cell monolayer was washed twice with phosphate-buffered saline and lysed by the addition of 4 ml of 0.05 M-Tris/HCl buffer, pH 7.4, containing 0.1 M-KCl, 0.01 M-EDTA and 0.5% sodium deoxycholate followed by two cycles of freezing and thawing. The lysate was centrifuged at 34000 rev./min (~100000 *g*) in a Kontron TST 54 rotor for 30 min at 4 °C, then stored at −20 °C.

Immunoprecipitation and SDS/polyacrylamide-gel electrophoresis

Culture supernatants or cell lysates corresponding to about 10^7 c.p.m. total were diluted with equal volumes of detergent buffer (1% Triton X-100, 0.5% sodium deoxycholate and 1% SDS in phosphate-buffered saline) containing 0.5% human serum albumin. The mixture was incubated at 4 °C overnight either with 5 μl of specific antibodies to $C\bar{1}s$ or with 20 μl of antiserum to $C\bar{1}r$ preincubated with 50 μl of C1-depleted serum for 4 h at 4 °C. Then 50 μl of IgG-Sorb, washed once with 1 ml of detergent buffer containing 0.5% human serum albumin, was added and the mixture incubated for 30 min at 4 °C. The pellets were collected by centrifugation, washed once with 1 ml of detergent buffer containing 0.5% human serum albumin and five to seven times with 1 ml of detergent buffer alone. After the final wash the pellets were resuspended in 50 μl of sample buffer (0.1 M-Tris/HCl buffer, pH 6.8, containing 2% SDS, 20% glycerol and 5% 2-mercaptoethanol) and boiled for 5 min, and the extract was analysed by SDS/polyacrylamide-gel electrophoresis.

SDS/10%-polyacrylamide-gel electrophoresis was carried out by the procedure of Laemmli (1970). ^{14}C-labelled M_r markers were included in each gel. After electrophoresis the gels were fixed by soaking overnight in 25% (w/v) trichloracetic acid, washed with water, impregnated with En^{3}Hance, dried and exposed to Kodak X-Omat AR films at −70 °C.

Radioimmunoassay of C1r and C1s

C1r and C1s were determined by a competitive radioimmunoassay as described by Bensa *et al.* (1983), with antibodies prepared in this laboratory and proteins purified and radiolabelled in this laboratory.

Sucrose-density-gradient ultracentrifugation

Samples were sedimented by the procedure of Martin & Ames (1961) in a linear 5–20% (w/v) sucrose gradient at 34000 rev./min in a TST 54 rotor in a Kontron TGA 50 ultracentrifuge for 15 h at 4 °C.

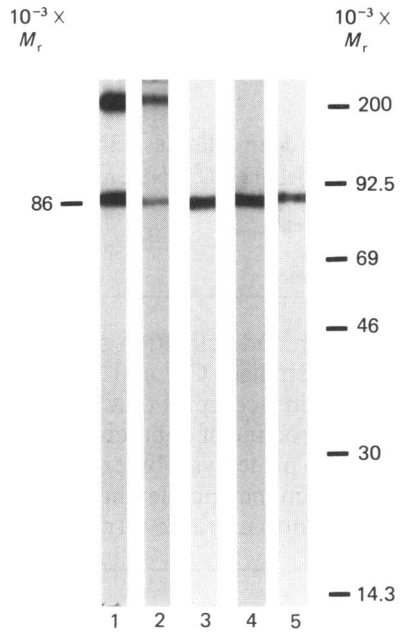

Fig. 1. Immunoprecipitation of C1r and C1s secreted by Hep G2 cells

Lanes 1 and 2, fluorographs after SDS/polyacrylamide-gel electrophoresis of C1r and C1s respectively immunoprecipitated from [^{35}S]methionine-labelled culture medium. Lanes 3 and 4, C1r and C1s respectively immunoprecipitated after fractionation of the culture medium by sucrose-density-gradient ultracentrifugation (Fig. 3) and corresponding to the C1r and C1s peaks. Lane 5, ^{125}I-labelled purified plasma C1s.

RESULTS

Characteristics of C1r and C1s synthesized by Hep G2 cells

Equivalent amounts of C1r and C1s were detectable by radioimmunoassay in supernatants of Hep G2 cell culture. For a monolayer established in a 25 cm^2 flask about 500 ng of each protein was secreted per 24 h. Lymphocyte-conditioned media, which we have shown to enhance C1r and C1s secretion by monocytes (Bensa *et al.*, 1983), were assayed on Hep G2 cells. Stimulation for 24 h by 5% (v/v) conditioned medium from staphylococcal-endotoxin-A-stimulated lymphocytes enhanced 5-fold the secretion of C1r and C1s, which reached 2 μg per 24 h for each protein.

Immunoprecipitation with anti-$C\bar{1}r$ serum or anti-$C\bar{1}s$ specific immunoglobulins of culture supernatants from cells continuously labelled for 17 h with L-[^{35}S]methionine is illustrated in Fig. 1. In lanes 1 and 2, corresponding to C1r and C1s respectively, two bands are clearly visible. The band of M_r approx. 86000 co-migrated with proenzymic ^{125}I-labelled $C\bar{1}s$ (Fig. 1, lane 5). The higher-M_r bands in the gels did not disappear after addition of excess unlabelled $C\bar{1}r$ or $C\bar{1}s$ before immunoprecipitation. To assess the identity of the 86000-M_r band the media were fractionated by sucrose-density-gradient ultracentrifugation (see the next paragraph) and the immunoprecipitation was made on fractions corresponding to the maximum of the protein peaks. In this case only the 86000-M_r peaks were

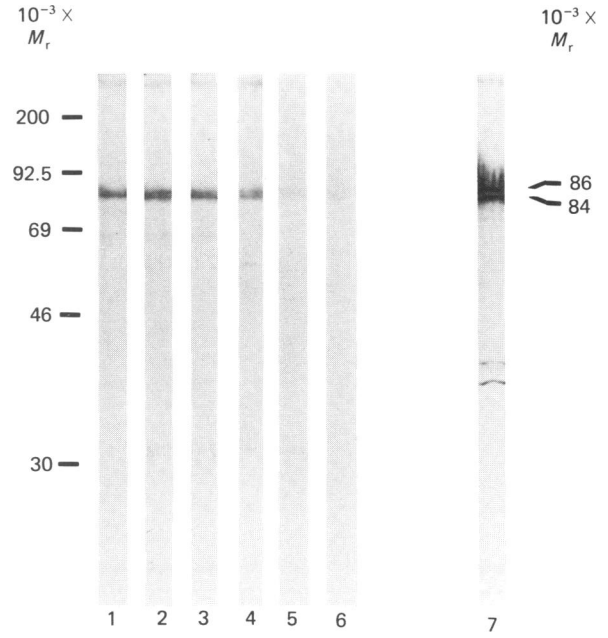

Fig. 2. Immunoprecipitation of Hep G2 intracellular C1s

Hep G2 cells were labelled with [^{35}S]methionine for 30 min and 'chased' with 0.25 mM unlabelled methionine for intervals up to 240 min. The cells were then solubilized and the lysates were immunoprecipitated and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Lanes 1–6, 'chase' times 0, 15, 30, 120 and 240 min respectively. Lane 7, immunoprecipitate of lysate from cells labelled with [^{35}S]methionine for 17 h at 23 °C.

observed in SDS/polyacrylamide-gel electrophoresis (Fig. 1, lanes 3 and 4).

It is worthwhile emphasizing that C1r and C1s were secreted as monomer proteins that, by SDS/polyacrylamide-gel electrophoresis, appeared to be identical with their proenzymic circulating counterparts; bands corresponding to A or B chains of activated $\overline{C1r}$ or $\overline{C1s}$ were never observed.

A 72000-M_r unidentified band, of variable intensity, was also sometimes revealed in reduced samples.

Cells were pulse-labelled for 30 min with L-[^{35}S]methionine and then 'chased' for different times with excess unlabelled methionine. A progressive decrease of intracellular radioactivity precipitated by anti-$\overline{C1r}$ or anti-$\overline{C1s}$ and a progressive accumulation in the culture supernatant was observed. In the case of C1r an intracellular protein of M_r approx. 84000 was immunoprecipitated in very small amounts. In the case of C1s the intracellular immunoprecipitated protein often appeared as a doublet (Fig. 2); similar results were obtained when accumulation of intracellular proteins was favoured by lowering the culture temperature from 37 °C to 23 °C (Fig. 2, lane 7). The bands of M_r approx. 200000 were not specific. The absence of specific larger-M_r intracellular proteins suggests that proprotein is unlikely to be present at any stage of C1r or C1s biosynthesis.

Sucrose-density-gradient centrifugation of Hep G2 cell culture supernatants

Hep G2 cell culture supernatants were analysed by sucrose-density-gradient centrifugation. C1r and C1s were measured in the eluted fractions by radioimmuno-

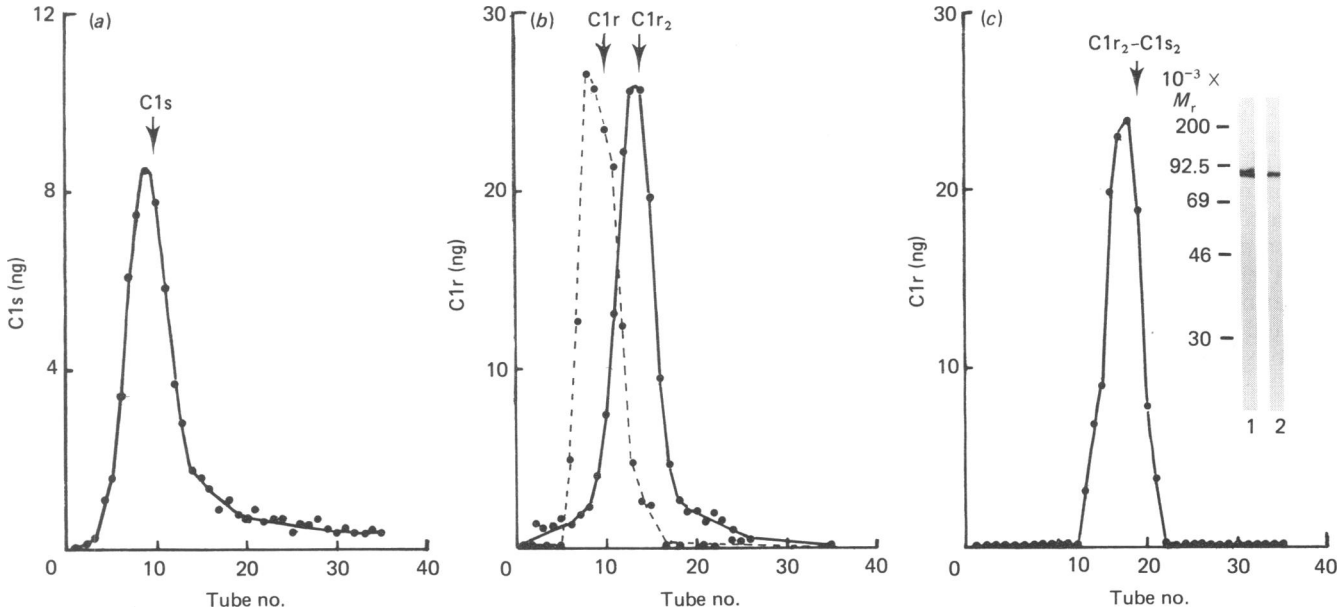

Fig. 3. Sucrose-density-gradient ultracentrifugation of Hep G2 cell culture supernatant

A 150 μl portion of cell culture supernatant was sedimented in a 5–20% sucrose density gradient as described in the Materials and methods section. Fractions were collected from the top of the gradient and analysed for C1r and C1s by radioimmunoassay. The arrows indicate the positions of the purified corresponding plasma proteins. (*a*) and (*b*, ——) Gradient in 0.145 M-NaCl/2.5 mM-EDTA/5 mM-sodium triethanolamine/HCl buffer, pH 7.4; (*b*, ------) gradient in 0.15 M-NaCl/1 mM-EDTA/30 mM-acetic acid/20 mM-glycine buffer, pH 5.0, the culture medium having previously been dialysed against the same buffer; (*c*) gradient in 0.145 M-NaCl/5 mM-$CaCl_2$/5 mM-sodium triethanolamine/HCl buffer, pH 7.4. The inset to (*c*) shows fluorograph of C1r (1) and C1s (2) immunoprecipitated from tube 17 after ultracentrifugation of [^{35}S]methionine-labelled cell culture supernatant.

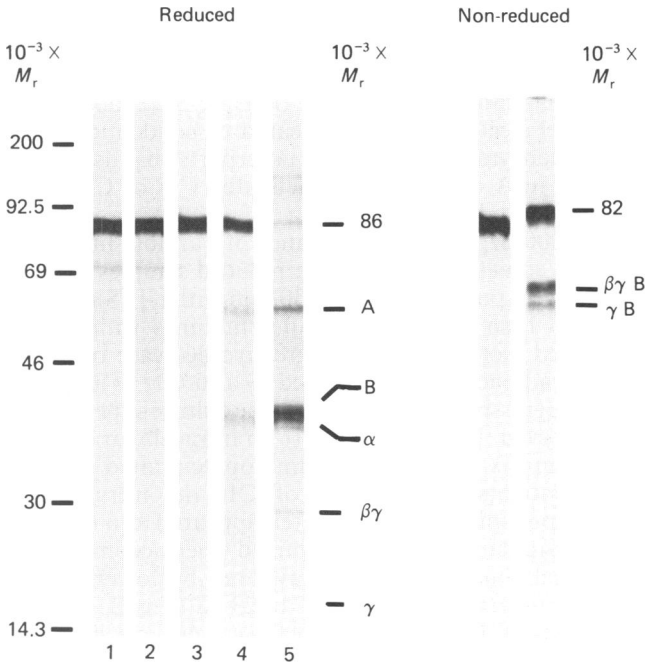

Fig. 4. Proteolysis of Hep-G2-cell-secreted C1r

[^{35}S]Methionine-labelled cell culture medium was fractionated by sucrose-density-gradient ultracentrifugation, and the immunoprecipitation was made directly on the peak of C1r (lanes 1 and 6, unreduced sample), after incubation of the peak for 1½ h at 37 °C (lane 2), after incubation of the peak for 1½ h at 37 °C with purified plasma $C\bar{1}r$ [10 ng (lane 3), 100 ng (lane 4) or 1 μg (lanes 5 and 7, unreduced sample]. The immunoprecipitates were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography.

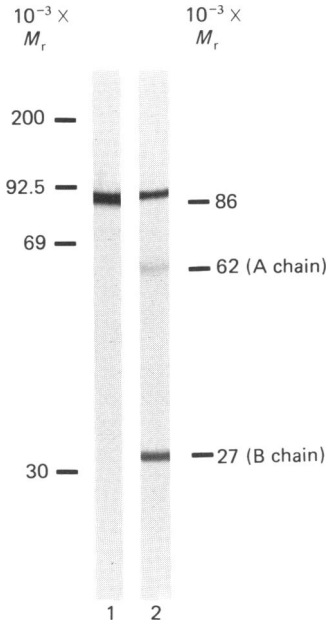

Fig. 5. Proteolysis of Hep-G2-cell-secreted C1s

[^{35}S]Methionine-labelled cell culture medium was fractionated by sucrose-density-gradient ultracentrifugation, and the immunoprecipitation was made directly on the peak of C1s (lane 1) or after incubation of the peak with 2 μg of purified plasma $C\bar{1}r$ for 30 min at 37 °C (lane 2). The immunoprecipitates were analysed by SDS/polyacrylamide-gel electrophoresis and fluorography.

assay, and their sedimentation coefficients were compared with those of $C\bar{1}r$ and $C\bar{1}s$ purified from plasma. In the different cases the observed sedimentations were comparable with although slightly slower than the sedimentations of the purified plasma proteins. When the gradient was prepared in an EDTA-containing buffer, secreted C1s migrated similarly to plasma $C\bar{1}s$ (Fig. 3*a*) and secreted C1r similarly to dimeric purified $C\bar{1}r$ (Fig. 3*b*). This last result points to the dimeric structure of secreted C1r. Lowering the pH to 5.0 induced a monomerization of secreted C1r (Fig. 3*b*). A similar influence of acidic pH was observed on the behaviour of purified $C\bar{1}r$ (Arlaud *et al.*, 1980).

The presence of Ca^{2+} in the gradient buffer resulted in a shift of the peak containing C1r to a position corresponding to $C1r_2$–$C1s_2$ complex (Fig. 3*c*); the simultaneous presence in the peak of C1r and C1s was confirmed by specific immunoprecipitation (Fig. 3*c* inset). These results clearly indicate the ability of secreted C1r and C1s to form a Ca^{2+}-dependent complex.

Susceptibility to proteolysis of secreted C1r and C1s

Supernatant from Hep G2 cell culture was fractionated on an EDTA-containing sucrose density gradient, and the tube corresponding to the peak of dimeric C1r was incubated at 37 °C for 1½ h before C1r immunoprecipitation. This treatment did not modify the electrophoretic behaviour of C1r, as evidenced by the persistence of a monomeric protein (Fig. 4, lanes 1 and 2). In contrast with C1r purified from plasma, secreted C1r did not support intrinsic autoproteolysis. To ensure that the lack of susceptibility to autoproteolysis was not due to the inhibitory effect of secreted $C\bar{1}$ Inh, $C\bar{1}$ Inh activity was blocked by an antiserum to $C\bar{1}$ Inh or by exogenous $C\bar{1}s$. The neutralization of $C\bar{1}$ Inh was not followed by autoproteolytic cleavage of secreted C1r (results not shown).

When, after fractionation of Hep G2 cell culture supernatant by sucrose-density-gradient centrifugation, the dimeric C1r peak was incubated with exogenous purified $C\bar{1}r$ before immunoprecipitation, two bands of M_r approx. 59000 and M_r approx. 37000 were visible on the electrophoretic pattern (Fig. 4, lane 5). The presence of these bands, which correspond to the A and B chains of activated C1r, indicated that secreted C1r has undergone extrinsic proteolysis by added $C\bar{1}r$. Moreover, other bands could be accounted for by a further proteolysis of activated secreted $C\bar{1}r$, leading to the generation of α (M_r approx. 35000), βγ (M_r approx. 24000) and γ (M_r approx. 16500) fragments, as has been described when purified $C\bar{1}r$ was incubated at 37 °C for several hours in the presence of EDTA (Assimeh *et al.*, 1978; Okamura & Fujii, 1978; Arlaud *et al.*, 1980). The electrophoretic pattern of non-reduced samples (Fig. 4, lanes 6 and 7) shows disulphide-bridged associations of these fragments at M_r approx. 61000 (βγ B) and M_r approx. 56000 (γ B). The extrinsic cleavage increased with the amount of added C1r, as indicated in Fig. 4 (lanes 3–5).

It is clearly apparent that secreted C1s is also susceptible to proteolysis by extrinsic $C\bar{1}r$. Incubation with $C\bar{1}r$ of the C1s peak, fractionated from Hep G2 cell

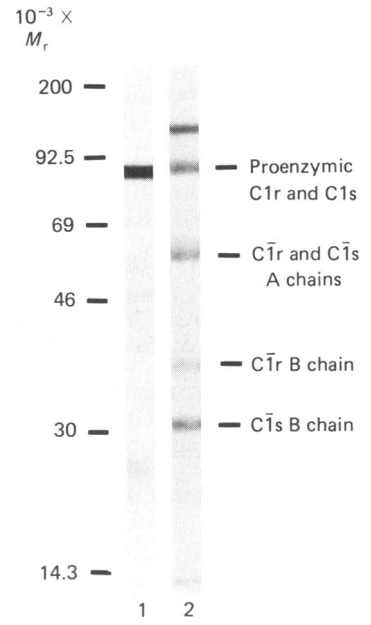

Fig. 6. Reconstitution of C1 with C1r and C1s secreted by Hep G2 cells

Immune-complex-bound C1q was prepared by incubating 1 mg of ovalbumin–anti-ovalbumin IgG aggregates with 60 μg of purified C1q for 40 min at 30 °C. The aggregates were washed with 110 mM-NaCl/2.5 mM-$CaCl_2$/20 mM-Tris/HCl buffer, pH 7.0, resuspended in 0.5 ml of [^{35}S]methionine-labelled Hep G2 cell culture supernatant supplemented with 2.5 mM-$CaCl_2$ and incubated for 40 min at 4 °C. The aggregates were washed with 110 mM-NaCl/2.5 mM-$CaCl_2$/20 mM-Tris/HCl buffer, pH 7.0, and incubated in the same buffer for 30 min at 30 °C. The aggregates were then extracted by 20 mM-EDTA/60 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.0, and the extract analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Lane 1, reconstitution with Hep G2 cell culture supernatant. Lane 2, reconstitution with Hep G2 cell culture supernatant in the presence of 30 μg of purified plasma $C\bar{1}r_2$–$C\bar{1}s_2$.

culture supernatant by sucrose-density-gradient centrifugation, followed by C1s immunoprecipitation, generated two fragments of M_r approx. 62000 and M_r approx. 27000 (Fig. 5), corresponding to the A and B chains of activated C1s.

Reconstitution of C1 from secreted C1r and C1s

The capacity of secreted C1r and C1s to reconstitute C1 was assayed by incubating immune-complex-bound C1q with Hep G2 cell culture medium in the presence of Ca^{2+}. The functionality of the reconstituted C1 was then assessed from the activation of C1r and C1s after incubation of immune-complex-bound C1 for 40 min at 30 °C and extraction by EDTA (Arlaud *et al.*, 1979).

Fig. 6 (lane 1) shows the electrophoretic pattern of the EDTA extract when C1 was reconstituted with Hep G2 cell culture supernatant pretreated with antiserum to C$\bar{1}$ Inh, to inhibit C$\bar{1}$ Inh activity. Most of the radioactivity extracted by EDTA migrated as a single band of M_r approx. 87000, identical with proenzymic C1r and C1s. When C1 was reconstituted with Hep G2 cell culture supernatant supplemented with purified $C\bar{1}r_2$–$C\bar{1}s_2$, the electrophoretic pattern of the EDTA extract clearly showed bands signifying C1r and C1s activation (Fig. 6, lane 2): bands of M_r approx. 60000 (C$\bar{1}$r and C$\bar{1}$s A chains), M_r approx. 37000 (C$\bar{1}$r B chain) and M_r approx. 28000 (C$\bar{1}$s B chain) were observed. These results show that at least part of secreted C1r and C1s is able to reconstitute C1 but that significant activation of C1 requires extrinsic $C\bar{1}r_2$–$C\bar{1}s_2$.

DISCUSSION

C1r and C1s are secreted by Hep G2 cells in stoichiometric amounts; in the presence of Ca^{2+} they are associated in a complex that sediments similarly to purified $C1r_2$–$C1s_2$. This type of association has already been found in serum, where circulating $C1r_2$–$C1s_2$ is loosely bound to C1q in C1. At physiological concentrations only about 70% of C1 subcomponents are associated (Ziccardi, 1983, 1984). Dissociation of C1 in C1q and $C1r_2$–$C1s_2$ can be achieved in the presence of citrate (Villiers *et al.*, 1982) or diamino compounds (Villiers *et al.*, 1984). Thus our results on biosynthesis confirm the tendency of $C1r_2$–$C1s_2$ to behave as a distinct entity in a 1:1 stoichiometry and may anticipate a genetic link between the corresponding genes.

C1q and $C1r_2$–$C1s_2$ biosyntheses appear to be distinct. Hep G2 cells do not synthesize detectable C1q. In contrast, in non-stimulated monocytes C1q biosynthesis largely predominates over C1r and C1s biosyntheses (Bensa *et al.*, 1983). Thus the balance between C1q, C1r and C1s should be controlled through the contribution of different cells. In the liver, for example, Kupfer cells, of monocytic origin, may collaborate with hepatocytes to allow the synthesis of whole C1.

Secreted C1r and C1s are able to reconstitute C1 in the presence of immune-complex-bound C1q. In fact, the radioactivity bound to immune-complex-bound C1q from radiolabelled Hep G2 cell culture supernatants is removable by EDTA.

C1r and C1s are secreted by Hep G2 cells in their non-activated form. As they are secreted as a Ca^{2+}-dependent association, this absence of activation most probably reflects the normal inhibition of C1r activation by associated C1s; in contrast with dimeric C1r, purified from plasma, which autoactivates itself, $C1r_2$–$C1s_2$ Ca^{2+}-dependent subcomponent was shown to be stable (Colomb *et al.*, 1984). Even when the Hep G2 cells secreted $C1r_2$–$C1s_2$ complex is dissociated by EDTA, C1r, in spite of its dimeric structure, appears to be unable to autoactivate itself. C$\bar{1}$ Inh does not seem to be involved in this inability. Slight structural differences could explain the different activation capacities of Hep-G2-cell-secreted C1r and its circulating counterpart. Autoactivation of purified dimeric C1r could be due to extracellular modifications, subsequent to secretion. Nevertheless, secreted C1r is proteolysed by exogenous C$\bar{1}$r. Secreted C1s is also proteolysed by exogenous C$\bar{1}$r; in this case the eventual contribution of secreted C1r cannot be estimated, as this C1r is likely to be activated by exogenous C$\bar{1}$r. The impaired activation of C1 reconstituted on immune complexes from secreted C1r and C1s could be related to a transient inability of secreted C1r to autoactivate itself. The apparent discrepancy between these results and previous reports on plasma C1r autoactivation could be explained by the very low

concentration of this protein among the different components synthesized by Hep G2 cells.

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