Structural Analysis of Clusterin and Its Subunits in Ram Rete Testis Fluid†

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ABSTRACT: Clusterin is a protein present in the rete testis fluid of the ram that elicits aggregation of erythrocytes and Sertoli cells in vitro. In view of its possible biologic function in relation to cell–cell interaction in the testis, we isolated this protein from ram rete testis fluid using sequential high-performance liquid chromatography columns and performed a detailed physicochemical characterization. This protein consists of two molecular variants designated form I and form II clusterin. Each form of clusterin consists of two subunits with an apparent molecular weight of 40 000. It is of note that the two subunits have no homology in their N-terminal amino acid sequences. However, the N-terminal amino acid pairs of the two subunits derived for the two forms of clusterin are identical. Using *o*-phthalaldehyde to block the Lys residue at the fourth amino acid pair from the N-terminus which leaves the Pro residue free for subsequent Edman degradation, we have deduced the N-terminal sequence of each of the two subunits for form I clusterin. Comparison of the NH_2-terminal sequences of the two subunits of clusterin with the release 10.0 of the protein sequence data base of the Protein Identification Resource indicated no homology between either of the subunits of clusterin and any of the known proteins in the data base. A highly specific radioimmunoassay developed for clusterin was used to measure its concentrations in the fluids of the rete testis and cauda epididymis. Since a significant amount of immunoreactive clusterin was found in serum, the protein was partially purified from this source by immunoaffinity chromatography. Immunoreactive serum clusterin was smaller than the testicular clusterin (M_r 37 000 vs 40 000), but both proteins share common epitopes as demonstrated by radioimmunoassay and immunoblots. However, serum clusterin does not possess the biologic activity of the testicular clusterin in that it does not elicit cell aggregation in vitro. It is of note that deglycosylation of testicular clusterin can also eliminate this in vitro biologic activity, suggesting that the serum clusterin might be a deglycosylated form of the testicular protein and the carbohydrate core plays an important role in determining the cell aggregation activity. Studies on the distribution of this protein in the reproductive compartment indicate that it is highly concentrated in the rete testis and the cauda epididymal fluids. This suggests that this protein might have some important functions in the reproductive tract.

Sertoli cells are the major secretory components of the seminiferous tubular epithelium, and they secrete the majority of their products into the adluminal compartment of the testis. Thus, these cells determine the protein composition of the seminiferous tubular fluid (Hansson et al., 1975; Mather et al., 1985; Bardin et al., 1981, 1988). Investigations from this and other laboratories indicate that Sertoli cells secrete both testis-specific and serum proteins (Wright et al., 1981; Wright & Luzarraga, 1986; Cheng et al., 1986; Cheng & Bardin, 1986, 1987; Kissinger et al., 1982; Shabanowitz et al., 1986; Kierszenbaum et al., 1986). Recently, one of the most abundant glycoproteins in the ram rete testis fluid was isolated (Blaschuk et al., 1983; Blaschuk & Fritz, 1984) and was designated clusterin on the basis of in vitro studies indicating that it could elicit aggregation of Sertoli cells from testes of immature rats, and erythrocytes from several species (Fritz et al., 1983). Clusterin was shown to be a dimer of M_r 80 000 consisting of two monomers of identical electrophoretic mobility of M_r 40 000 (Blaschuk et al., 1983). Immunohistochemical studies showed that clusterin is localized in the seminiferous tubule in a pattern consistent with its localization in Sertoli cells (Tung & Fritz, 1985). Since the studies of Sertoli cell proteins can provide insights into the physiology of seminiferous tubule, we thought it pertinent to perform a detailed structural analysis of this protein and to set up a specific radioimmunoassay to determine the distribution of this protein in the biological fluids of the ram.

Experimental Procedures

Materials

Biochemicals. $Na^{125}I$ (specific activity 15.1–17.1 mCi of $^{125}I/\mu g$ of iodine; code IMS 300) was obtained from Amersham (Arlington Heights, IL). Tris(hydroxymethyl)aminomethane (Tris),[1] acrylamide, *N*,*N*′-diallyltartardiamide (DATA), *N*,*N*′-methylenebis(acrylamide) (Bis), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulfate, agarose (electrophoresis grade), prestained high molecular weight marker proteins, and *Staphylococcus aureus* (formalin fixed) used as a source of protein A for immunoprecipitation were from Bethesda Research Laboratories (Gaithersburg, MD). Sodium chloride, citric acid, trifluoromethanesulfonic

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[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; HPLC, high-performance liquid chromatography; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; RIA, radioimmunoassay; NPB buffer, 10 mM sodium phosphate, pH 7.4 at 22 °C; PBS buffer, 10 mM sodium phosphate/0.15 M NaCl, pH 7.4 at 22 °C; *T*, total acrylamide concentration (g/100 mL), [acrylamide] + [methylenebis(acrylamide)]; C_{DATD}, amount of cross-linker using *N*,*N*′-diallyltartardiamide (DATD); % *C*, percent cross-linker, 100 × {[methylenebis(acrylamide)]/([acrylamide] + [methylenebis(acrylamide)])}; Bis, *N*,*N*′-methylenebis(acrylamide); BSA, bovine serum albumin; PTH, phenylthiohydantoin.

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acid, pyridine, diethyl ether (anhydrous), anisole, and silver nitrate were from Aldrich (Milwaukee, WI). Methanol, hydrogen peroxide (30% v/v), and formaldehyde solution (37% w/v) were from Fisher (Fair Lawn, NJ). Methanol (HPLC grade) was from Burdick & Jackson Laboratories, Inc. (Muskegon, MI). Glycine, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), DEAE-Bio-Gel A, and high and low molecular weight protein standards were from Bio-Rad (Richmond, CA). Protein A–peroxidase, wheat germ agglutinin (WGA)–peroxidase, 4-chloro-1-naphthol, concanavalin A, bovine serum albumin (fraction V), NP-40 [Nonidet P-40, octylphenoxypoly(ethoxyethanol)], neuraminidase (*Clostridium perfringens*), β-galactosidase (*Aspergillus niger*), β-*N*-acetylglucosaminidase (*Aspergillus niger*), and Cibacron blue–Sepharose 4B were obtained from Sigma (St. Louis, MO). α-Mannosidase (*Turbo cornutus*) was from Miles Laboratories, Inc. (Elkhart, IN). Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril), *o*-phthaladehyde, and acetic acid were from Pierce (Rockford, IL). Normal rabbit serum (lyophilized) was from Calbiochem (La Jolla, CA). Tween 20 [poly(oxyethylene)-sorbitan monolaurate], Coomassie blue G-250, Coomassie blue R-250, and BSA (RIA grade) were from United States Biochemical Corp. (Cleveland, OH). *Staphylococcus aureus* protease V_8 was from Miles Laboratories, Inc. (Elkhart, IN). Nitrocellulose paper (0.15- and 0.45-μm pore size) was from Schleicher & Schuell, Inc. (Keene, NH).

HPLC Columns. A Mono Q (HR 5/5) anion-exchange HPLC column (5 × 50 mm i.d., particle size 10 μm) was obtained from Pharmacia. A Bio-Sil TSK-250 gel permeation HPLC column (7.5 × 300 mm i.d.) and a Bio-Sil guard column (7.5 × 75 mm i.d.) were from Bio-Rad.

Animals and Tissue. Rete testis fluid and cauda epididymal fluid were collected from Rambouillet rams aged between 3 and 6 years of age and weighing between 50 and 70 kg as previously described (Voglmayr & White, 1979; Voglmayr et al., 1980).

Methods

Analytical Polyacrylamide Gel Electrophoresis (PAGE). Analytical PAGE in the presence of SDS was performed by the method of Laemmli (1970). The resolving gel consisted of either 10% or 12.5% *T* (total acrylamide concentration) and 2.6% cross-linker using methylenebis(acrylamide) (% C_{Bis}) with a stacking gel of 5% *T* and 15% *N*,*N*′-diallyltartardiamide (% C_{DATD}). For peptide mapping (Cleveland et al., 1977), the resolving gel consisted of 15% *T* and 2.6% C_{Bis} while the stacking gel was the same as above. Gels were stained either with silver nitrate (Wray et al., 1981) or with Coomassie blue R-250 (Cheng et al., 1983).

General Methods. ^{125}I-Clusterin was quantified by γ scintillation spectrometry (Packard, Model 5110) at 70% counting efficiency. Protein concentration was estimated by the dye binding method of Bradford (1976) as modified by Macart and Gerbaut (1982) using BSA as a standard. Electrophoretic transfer, immunoblots, and lectin blots were performed by established procedures (Burnette, 1981; Hawkes, 1982) as previously described (Cheng et al., 1985a,b; Cheng & Bardin, 1986). Peptide mapping using protease V_8 was performed as described by Cleveland et al. (1977) as detailed elsewhere (Cheng et al., 1985a,b; Cheng & Bardin, 1986). Crossed- and tandem crossed-immunoelectrophoreses were performed according the procedures of Weeke (1975).

Purification of Clusterin. (A) Preparation of Ram Rete Testis Fluid for HPLC. For each of the six different experiments, about 50–150 mL of ram rete testis fluid was used. Fluids were thawed, concentrated, desalted, and equilibrated against 20 mM Tris, pH 7.4 at 22 °C, in an Amicon ultrafiltration unit (Model 8050) and a YM-10 membrane (molecular weight cutoff at 10000). This and all subsequent procedures, unless otherwise specified, were performed at 4 °C. The desalted sample was concentrated to about 5 mL and filtered through a 0.45-μm filter unit (Uniflo from Schleicher & Schuell).

(B) Anion-Exchange HPLC of Proteins in Ram Rete Testis Fluid. The procedures for the fractionation of proteins contained in the ram rete testis fluid were essentially as previously described (Cheng & Bardin, 1986). Briefly, samples were loaded onto the Mono Q column at a flow rate of 1 mL/min using solvent A consisting of 20 mM Tris, pH 7.4 at 22 °C. Bound proteins were then eluted with a linear salt gradient from 0 to 80% solvent B consisting of 20 mM Tris and 600 mM NaCl, pH 7.4 at 22 °C. Fractions were collected, and the effluent was monitored by the UV absorbance at 280 nm.

(C) Cibacron Blue F3GA–Sepharose Chromatography. Fractions containing clusterin obtained from the anion-exchange HPLC were pooled, concentrated, and desalted against 0.05 M Tris, pH 8.0, containing 50 mM NaCl using an Amicon ultrafiltration cell. The sample was then applied to a Cibacron blue F3GA–Sepharose column of 1.5 × 10 cm as previously described (Travis et al., 1976). Fractions (1 mL each) were collected, and samples containing clusterin were pooled and concentrated to about 200 μL using a Centricon-10 microconcentrator for further purification. During this step, essentially all the albumin-like protein contained in the previous step was removed which remained specifically bound to the resin.

(D) Gel Permeation HPLC. The concentrated clusterin sample was injected onto a gel permeation HPLC column (Bio-Sil TSK-250, 7.5 × 300 mm i.d.) using a 200-μL sample loop as previously described (Cheng & Bardin, 1986). Proteins were eluted with 10 mM sodium phosphate/0.15 M NaCl, pH 6.8 at 22 °C, at a flow rate of 0.5 mL/min.

In Vitro Bioassay of Clusterin. Ram rete testis fluid and both forms of purified clusterin were assayed for clusterin activity as previously described (Fritz et al., 1983) with minor modifications. Briefly, 0.5 mL of blood was withdrawn from a 60-day-old Sprague-Dawley rat by cardiac puncture under light ether anesthesia and suspended immediately in 5 mL of PBS buffer (10 mM sodium phosphate/0.15 M NaCl, pH 7.4 at 22 °C). Erythrocytes were washed twice at 500 g and reconstituted in 1 mL of PBS buffer. The assay was performed at room temperature. The cell suspension was diluted with PBS buffer, and a total of 25 μL containing about 4.5×10^6 cells was added to each of the 96-well Immulon-2 flat-bottom plates from Dynatech Laboratories Inc. (Alexandria, VA). Ram rete testis fluid, partially purified clusterin from different steps of the purification scheme, and purified clusterin at different dilutions were added to the wells to a final volume of 100 μL. All assays were done in duplicate. A set of positive controls was included in each assay using serially diluted ram rete testis fluid. Negative controls were performed using PBS buffer and purified carbonic anhydrase at concentrations ranging between 1 and 70 μg per assay well. To determine the effect of deglycosylation on clusterin in vitro biologic activity, clusterin following enzymatic and chemical deglycosylation was incubated with erythrocytes at various concentrations ranging between 0.05 and 0.5 μg per assay well. Control incubations were prepared by using ovalbumin treated either with a mixture of exoglycosidases or with trifluoromethanesulfonic acid. The plates were then incubated at room temperature for 12 h on a Red Rocker (Model PR 50) from

Hoefer and examined by phase-contrast microscopy to determine the clusterin activity. It is of note that it routinely took as little as 0.5 μL of ram rete testis fluid or 0.05 μg of purified clusterin to induce cell aggregation in a 0.1-mL suspension of erythrocytes.

Preparation of Clusterin Antiserum. Purified clusterin (70 μg in 0.5 mL of NPB buffer) was emulsified with an equal volume of Freund's complete adjuvant and injected intradermally into a New Zealand White female rabbit in multiple sites. Six weeks later, the rabbit received a booster injection of clusterin (70 μg in 0.5 mL of NPB buffer) emulsified with an equal volume of Freund's incomplete adjuvant and was bled 10 days after the boost. The blood was allowed to clot at 4 °C overnight and the serum obtained by centrifugation at 2000*g* for 10 min at 4 °C. Several bleedings were tested individually, and the antiserum used for this study represented the highest titer generated. For radioimmunoassay, the antiserum was diluted in BSA–PBS buffer (10 mM sodium phosphate/0.15 M NaCl, pH 7.4 at 22 °C, containing 0.5% BSA) containing 2% normal rabbit serum. A working dilution of 1:1500 was the selection for RIA which gave a 25% binding and represented a final dilution of 1:7500.

Radioimmunoassay of Clusterin. Clusterin was iodinated by Iodogen using established procedures (Fraker & Speck, 1978). The labeled protein was separated from the free iodide by Sephadex G-50 and DEAE-Bio-Gel A chromatography as detailed elsewhere (Cheng et al., 1983; Cheng & Bardin, 1986). A pool of ram rete testis fluid was used as a laboratory reference standard to estimate the concentrations of clusterin in unknown samples. When this pool (CYCRTF-1) was compared to a highly purified clusterin preparation (CYCRTF3-III) using a dye binding assay (Macart & Gerbaut, 1982), it was found that 1 μL of CYCRTF-1 contained 97 ng of purified clusterin. For radioimmunoassay, each assay tube contained 200 μL of BSA–PBS buffer, 100 μL of standard or sample diluted in BSA–PBS buffer, 100 μL of antiserum at a working dilution of 1:1500, and 100 μL of radiolabeled clusterin (about 10 000 cpm) to give a final volume of 500 μL. The assay mixture was vortexed briefly and then incubated at 4 °C for 36 h. Thereafter, the bound clusterin and free iodinated clusterin were separated by adding 30 μL of formalin-fixed *Staphylococcus aureus* cells as previously described (Cheng & Bardin, 1986). Each standard or sample was run in duplicate or triplicate and analyzed as previously described (Cheng et al., 1983). The intraassay and interassay coefficient of variation was 10% and 15%, respectively. The minimal detectable dose was 0.0015 μL equiv/assay tube, and 50% displacement was at 0.04 μL equiv.

Amino Acid Analysis. Purified clusterin was hydrolyzed in 6 N HCl plus 0.1% phenol at 110 °C for 22 h. Analyses were performed using an automated Waters Pico-tag work station (Millipore Corp., Milford, MA) with a Pico-tag column (3.9 mm × 150 mm i.d.) operated at 38 °C.

Preparation of Clusterin for N-Terminal Sequence Analysis Using Edman Degradation. Amino acid sequencing was performed with an Applied Biosystems Model 470A gas-phase analyzer on 0.1–1-nmol samples of intact clusterin. The protein was equilibrated and concentrated in a 0.1% SDS solution using a Centricon-10 microconcentrator from Amicon operated at 4 °C using a JA20.1 rotor head at 4640*g*. The entire concentration and equilibration step routinely took 2–4 h to complete. The detergent was used as a protein solubilizer and minimized adsorption of protein to glass or plastic. The sample solution was concentrated to about 100 μL which contained between 0.1 and 0.8 nmol of purified clusterin. Phenylthiohydantoin (PTH)-amino acids were identified and quantitated by reverse-phase HPLC. In the experiments for sequencing each subunit of clusterin in the presence of the other, *o*-phthalaldehyde treatment was applied prior to cycles in which proline was present, according to a procedure as previously described (Brauer et al., 1984; Gross et al., 1985) in which the NH_2 terminus of the other amino acid was blocked, leaving the subunit with proline freely accessible for subsequent Edman degradation. The *o*-phthalaldehyde dissolved in *n*-butyl chloride (0.2 mg/mL) was manually applied in a total volume of 30 μL containing dithiothreitol at 0.2 mg/mL. All subsequent procedures including sequencer program cycles were as previously described (Gross et al., 1985). PTH-amino acids were determined by using a Hewlett-Packard liquid chromatography terminal (Model 1084A) equipped with a two-pump gradient system and an autosampler. A Du Pont Zorbax ODS HPLC column (4.6 × 250 mm i.d.) was operated at 50 °C, and the elution was monitored at 254 nm using a flow rate of 1.35 mL/min with a gradient of 0–100% buffer B in 45 min. Buffer A consisted of 0.05 M sodium acetate, pH 5.3 at 40 °C, and buffer B consisted of 100% acetonitrile. The repetitive yield was about 95%, and the percent blockage using *o*-phthalaldehyde was greater than 98%.

Partial Purification of Clusterin from Ram Plasma by Immunoaffinity Chromatography. A pool of 50 mL of ram plasma was used as a source of ram clusterin. Rabbit anti-ram clusterin IgG was purified from the crude antiserum by sequential ammonium sulfate precipitation and DEAE-Bio-Gel A chromatography essentially as previously described (Livingston, 1974). The purity of the IgG was confirmed by SDS–PAGE. The purified anti-ram clusterin IgG was conjugated to Sepharose 4B using tresyl-activated Sepharose (Pharmacia) with a ligand:resin ratio of 8 mg protein to 1 mL of resin. The coupling reaction occurred by nucleophilic displacement of the active tresyl (2,2,2-trifluoromethanesulfonyl chloride) groups by both the NH_2 and SH groups of the ligand to form stable covalent bonds between the ligand and the matrix. The reaction proceeded in the coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl, pH 8 at 22 °C) for 24 h at 4 °C. The excessive ligand was washed away with the coupling buffer, and any remaining active groups were blocked with 0.1 M Tris buffer (pH 8 at 22 °C) for 4 h at 4 °C. The column was then washed sequentially with 10 column volumes of 50 mM Tris, pH 8 at 22 °C, containing 0.5 M NaCl, with 50 mM sodium acetate, pH 4.5 at 22 °C, containing 0.5 M NaCl, and with 20 mM Tris, pH 7.4 at 22 °C. Ram plasma was then pumped onto the immunoaffinity column (1.5 × 15 cm) and recirculated through the affinity matrix at a flow rate of 10 mL/h for 48 h to allow the immunoreactive clusterin to interact with the matrix. Unbound proteins were then eluted with 20 mM Tris, pH 7.4 at 22 °C, containing 0.5 M NaCl. Immunoreactive clusterin that was specifically bound to the affinity gel was eluted with 0.1 M glycine buffer (pH 2.5 at 22 °C). The pH of the eluates was immediately adjusted to pH 7.5 with 2 M Tris, and the samples were concentrated and desalted using an Amicon concentrator.

Enzymatic Deglycosylation of Ram Testicular Clusterin. Enzymatic and chemical deglycosylation of purified testicular clusterin was performed by using procedures as previously described (Cheng et al., 1985a,b; Cheng & Bardin, 1987). For enzymatic deglycosylation, 20–30 μg of immunoreactive clusterin was equilibrated in 0.1 M sodium acetate buffer, pH 5.0 at 22 °C, in a Centricon-10 microconcentrator from Amicon. Proteins were incubated with a mixture of exo-

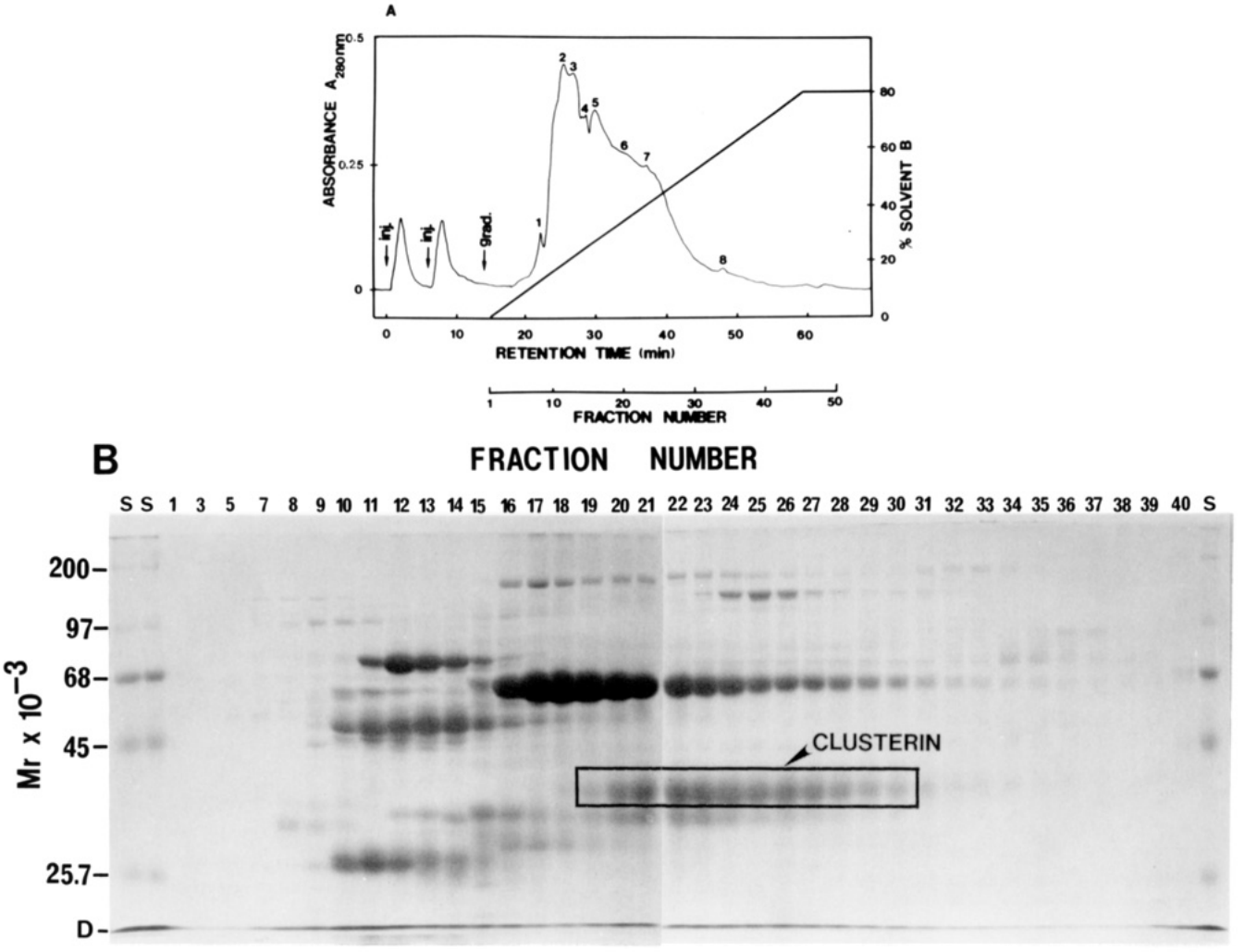

FIGURE 1: Fractionation of ram rete testis fluid for the isolation of clusterin. (A) Elution profile of protein in rete testis fluid from anion-exchange HPLC. A pool of ram rete testis fluid (50-mL sample consisted of a total protein content of about 35 mg) was equilibrated against solvent A (20 mM Tris, pH 7.4 at 22 °C) in Spectraphor dialysis tubing with a molecular weight cutoff of 6000. Samples were then concentrated to about 4 mL by using an Amicon ultrafiltration unit (Model 8010), filtered through a Uniflo filtering unit (0.45 μm; Schleicher & Schuell, Kleene, NH), and loaded onto a Mono Q column (5 × 50 mm i.d.) using a flow rate of 1 mL/min. Bound proteins were eluted by using a linear gradient of 0–80% solvent B (20 mM Tris, pH 7.4 at 22 °C, containing 600 mM NaCl) over a period of 45 min at a flow rate of 1 mL/min. Proteins were monitored by the UV absorbance at 280 nm. About eight protein peaks were distinguished. (B) An aliquot from each fraction obtained in (A) was resolved by SDS–PAGE on a 10% *T* gel and stained with Coomassie blue. Clusterin was eluted between fractions 19 and 30. S indicates the lanes for BRL prestained molecular weight markers consisting of myosin, phosphorylase *b*, BSA, ovalbumin, and chymotrypsinogen. D, dye front.

glycosidases including neuraminidase, β-galactosidase, α-mannosidase, and β-*N*-acetylglucosaminidase for 36 h at 37 °C using 0.05 unit of each of the enzymes per 100 μg of protein. Samples were then equilibrated in PBS buffer using a Centricon-10 microconcentrator and serially diluted in the PBS buffer at concentrations ranging between 0.05 and 0.5 μg per assay well for the in vitro bioassay to monitor the clusterin activity. Control samples were prepared by using ovalbumin treated with a mixture of exoglycosidases identical with the clusterin samples. For chemical deglycosylation, purified ram clusterin was treated with trifluoromethanesulfonic acid as previously described (Edge et al., 1981; Cheng et al., 1985a; Cheng & Bardin, 1987). Purified clusterin (about 50 μg of protein) was equlibrated in double-distilled water on a Centricon-10 microconcentrator, lyophilized, and resuspended in 90 μL of anhydrous trifluoromethanesulfonic acid and 18 μL of anisole. The sample was transferred to a Reacti-vial from Pierce and incubated at room temperature with agitation for 6 h. Thereafter, the reaction mixture was mixed with 1 mL of pyridine/diethyl ether (1:9 v/v) in a dry ice/methanol bath. The protein and pyridinium salt of the acid were coprecipitated and were collected by centrifugation at 10000*g* for 15 min at 4 °C. The pellet was resuspended in 0.1 M NH_4HCO_3 and dialyzed extensively against this solution. The neutralized, chemically deglycosylated clusterin was then resuspended and equilibrated against PBS buffer on a Centricon-10 microconcentrator. Samples were analyzed by immunoblots, by lectin blots, and by in vitro bioassay.

RESULTS

Purification of Clusterin from Ram Rete Testis Fluid. Each batch of ram rete testis fluid was fractionated by using the purification scheme outlined in Table I, and it has been repeated 6 times over a period of 3 years. The preparation reported in Table I had a total protein content of about 35 mg of which 4.8 mg was shown to be clusterin by radioimmunoassay. Thus, clusterin contributed about 10% of the total protein content in the ram rete testis fluid. When this pool of rete testis fluid was fractionated onto a Mono Q column, at least eight protein peaks were detected as shown in Figure 1A. Clusterin was shown to elute in fractions 19–30 by using radioimmunoassay and SDS–PAGE; its apprent molecular

Table I: Summary of the Purification Scheme for Clusterin from Ram Rete Testis Fluid

step	[clusterin][a] (nmol)	[protein][b] (mg)	sp act.[c]	cumulative purification: from previous step	cumulative purification: cumulative	recovery (%): from previous step	recovery (%): cumulative
pooled ram rete testis fluid (50 mL)	60	35	1.7		1		100
anion-exchange HPLC	44	10	4.4	2.6	2.6	73	73
Cibacron blue–Sepharose 4B	28	4.2	6.7	1.5	3.9	64	47
gel permeation HPLC[d]							
form I clusterin	13	0.9	14	2.2	8.5	46	22
form II clusterin	10	0.8	13	1.9	7.4	36	17

[a] The clusterin concentration was measured by radioimmunoassay using an apparent molecular weight of 80 000. [b] The protein concentration was determined by the dye binding assay with bovine serum albumin as a standard as described under Experimental Procedures. [c] Specific activity was expressed as nanomoles of clusterin per milligram of protein. [d] In this step, the two forms of clusterin were separated due to the differential interactions between the carbohydrate moiety of the protein with the column matrix. Thus, the lower yield for each form of clusterin in this step did not reflect low recovery from the column.

weight was estimated to be 40 000 (Figure 1A,B). These fractions were then concentrated and fractionated on a Cibacron blue F3GA–Sepharose column. Clusterin did not bind to this dye affinity column; however, the major 68K contaminant bound to the resin and was thus removed. The partially purified clusterin sample was then fractionated on a gel permeation HPLC column as shown in Figure 2A; six protein peaks were observed. When these fractions were resolved by SDS–PAGE as shown in Figure 2B, proteins of apparent molecular weight of 40 000 with properties of clusterin were eluted in peaks 2 and 3 (Figure 2A,B). These two peaks of clusterin were tentatively designated form I and form II for peaks 2 and 3, respectively, in Figure 2A. Form I clusterin was used to prepare a monospecific polyclonal antiserum against this protein. Analysis of the purified proteins by SDS–PAGE (Figure 2B) and the specific activity (Table I) indicated that both forms of clusterin had been purified to apparent homogeneity.

Physicochemical and Immunological Characterization of Clusterin. Apparent Molecular Weight of Clusterin. The apparent molecular weights of purified form I and form II clusterin determined by gel peremation HPLC using a Bio-Sil TSK-250 column (7.5 × 600 mm i.d.) (Cheng & Bardin, 1986, 1987) were 86 000 and 82 000, respectively. The apparent molecular weights of both forms of clusterin estimated under reducing conditions by SDS–PAGE were approximately 40 000.

Characterization of the Two Forms of Clusterin. (A) Immunoblots and Immunoelectrophoresis. When crude ram rete testis fluid, form I clusterin, and form II clusterin were fractionated by SDS–PAGE and subsequently immunostained with the form I clusterin antiserum, only one protein band was detected with a molecular weight of 40 000 in each sample (Figure 3, lanes 1 and 2) that corresponded to the Coomassie blue stained protein (Figure 2B). However, purified form II and form I clusterin (Figure 3, lanes 3 and 4, respectively) had minor differences in their electrophoretic mobility on SDS–PAGE at lower gel concentration (7.5% *T*) which were undetectable with either 10% or 12.5% *T* gels shown in Figures 1B and 2B. Examination of crude ram rete tetis fluid by crossed-immunoelectrophoresis using anti-form I clusterin antiserum revealed only one immunoprecipitation arc (Figure 4A), whereas when both forms of clusterin were resolved by tandem crossed-immunoelectrophoresis, two completely fused immunoprecipitation arcs were observed; these observations suggest that form I clusterin and form II clusterin share common epitopes (Figure 4B).

(B) Amino Acid Composition Analysis. Both forms of clusterin were hydrolyzed to their respective amino acids using 6 N HCl containing 0.1% phenol, and their amino acid composition was determined by reverse-phase HPLC. Two separate analyses were performed on each form of clusterin, and the results are summarized in Table II. These results indi-

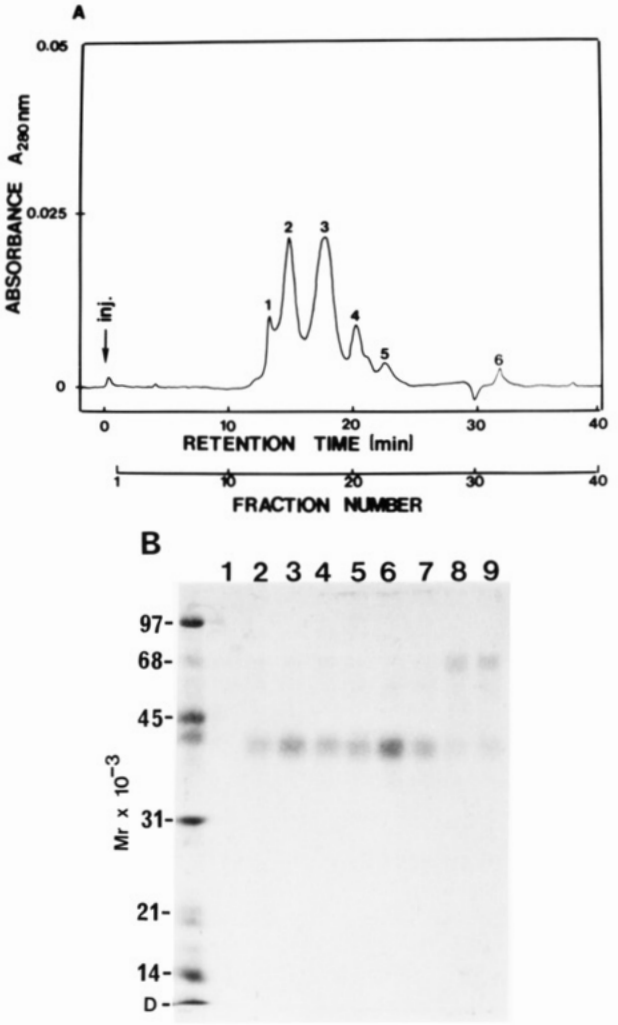

FIGURE 2: Purification of ram clusterin by gel permeation HPLC. (A) Partially purified clusterin eluted in the nonbound fractions of the Cibacron blue F3GA–Sepharose 4B column was concentrated and equilibrated against 10 mM sodium phosphate/0.15 M NaCl, pH 6.8 at 22 °C. The sample was injected onto a gel permeation HPLC column (Bio-Sil TSK 250). Six protein peaks were observed. (B) An aliquot from each of the fractions shown in (A) was resolved by SDS–PAGE on a 12.5% *T* gel and stained with Coomassie blue. Also, these fractions were screened for clusterin activity by using both immunoblots and the in vitro biologic assays. Lanes 1–9 corresponded to fractions 13–21, respectively. Form I clusterin was eluted in fractions 14 and 15 [lanes 2 and 3, peak 2 in (A)], and form II clusterin eluted in fractions 18 and 19 [lanes 6 and 7, peak 3 in (A)].

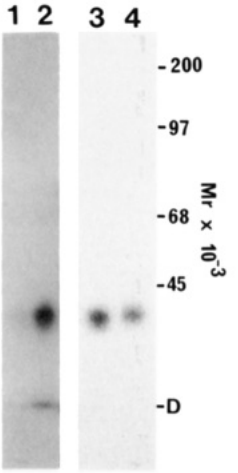

FIGURE 3: Immunoblots of ram rete testis fluid and purified clusterin. Crude ram rete testis fluid (lanes 1 and 2 corresponded to 0.05- and 0.5-μL samples, respectively) and purified form II (lane 3, consisting of about 50 ng of protein) and form I (lane 4, consisting of about 40 ng of protein) clusterin were resolved on a 7.5% *T* SDS–polyacrylamide gel, transferred onto nitrocellulose paper, and stained with form I clusterin antiserum.

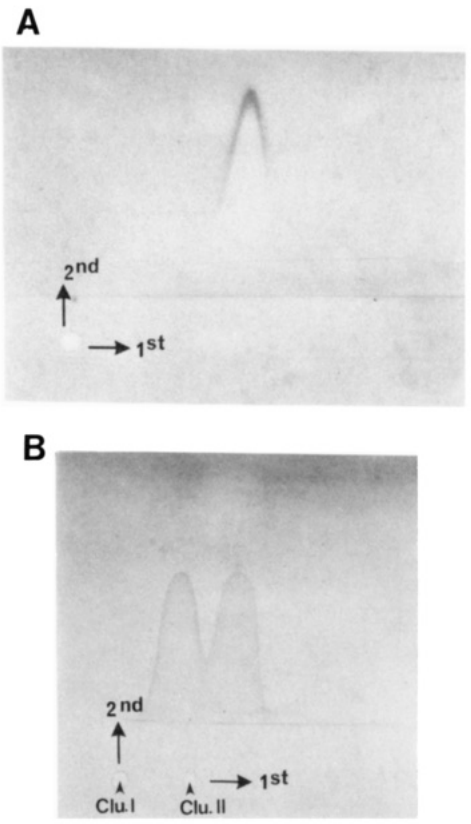

FIGURE 4: Immunoelectrophoresis of clusterin. Samples were resolved in the first dimension on a 1% agarose gel at 100 V running at 15 °C; the second dimension was performed on a 1% agarose gel contained 1% anti-clusterin antiserum at 50 V. (A) Crossed-immunoelectrophoresis of crude ram rete testis fluid. (B) Tandem crossed-immunoelectrophoresis of form I and form II clusterin using anti-form I clusterin antiserum.

cated that it was not possible to distinguish form I clusterin from form II by the amino acid composition (Table II). The amino acid composition reported for form I and form II clusterin is similar to previously published results for clusterin (Blaschuk et al., 1983) which might represent a mixture of both forms.

(*C*) *Peptide Maps.* Peptide fragments were generated by using *Staphylococcus aureus* protease V_8 which specifically

Table II: Amino Acid Composition of the Two Forms of Clusterin[a]

amino acid	clusterin (g/100 g of protein) form I	clusterin (g/100 g of protein) form II	amino acid	clusterin (g/100 g of protein) form I	clusterin (g/100 g of protein) form II
Asp/Asn	9.8	10.1	Pro	5.1	5.0
Glu/Gln	12.1	13.7	Tyr	3.0	2.7
Ser	11.2	10.9	Val	5.1	5.0
Gly	6.0	5.0	Met	2.0	2.4
His	2.6	2.5	Ile	2.8	2.6
Arg	5.5	5.0	Leu	9.1	9.6
Thr	7.0	6.8	Phe	4.8	4.9
Ala	6.2	5.8	Lys	5.6	6.2

[a] 0.2 nmol of clusterin was hydrolyzed in an evacuated tube containing 6 N HCl and 0.1% phenol for 22 h at 110 °C. It is of note that Cys was destroyed in the presence of phenol. Results represent the mean of two separate determinations.

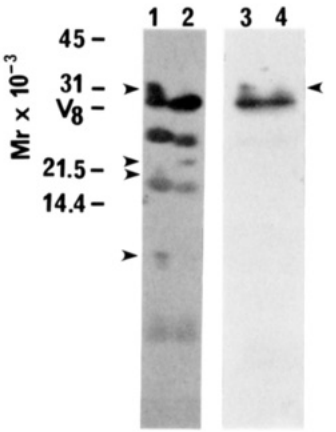

FIGURE 5: Peptide maps of the two forms of clusterin. Proteins were digested with protease V_8 as described under Experimental Procedures and fractionated on a 15% *T* SDS–polyacrylamide gel, transferred onto nitrocellulose paper, and stained with either anti-clusterin antibody (lanes 1 and 2) or WGA (lanes 3 and 4). Lanes 1 and 3 were form I clusterin, and lanes 2 and 4 were form II clusterin. Arrows indicate unmatched peptides.

cleaves peptide bonds on the carboxy-terminal side of either glutamate or aspartate residues (Drapeau et al., 1972). The peptide fragments were fractionated on a 15% *T* SDS–polyacrylamide gel, transferred onto nitrocellulose paper, and visualized using the anti-form I clusterin antiserum. It was of note that both forms of clusterin were very similar (Figure 5, lanes 1 and 2). The minor differences observed in their fragmentation pattern could be the results of differential glycosylation. When these fragments were visualized with a lectin blot using WGA–peroxidase which is specific for *N*-acetylglucosaminyl residues, form I clusterin demonstrated an extra peptide fragment that bound WGA (Figure 5, lanes 3 and 4). Neither form of clusterin bound concanavalin A either before or after proteolysis. These observations in conjunction with the amino acid composition and NH_2-terminal sequence analysis (see below) suggest that the two forms of clusterin are virtually identical proteins but each has different carbohydrate constituents which could be the basis of the differential interactions with the gel permeation HPLC column as shown in Figure 2A.

(*D*) *NH_2-Terminal Sequence Analysis of Form I and Form II Clusterin and Their Subunits.* The NH_2 termini of the two forms of clusterin were determined and found to be identical for the first 25 amino acids. Each form of clusterin, however, consisted of two dissimilar subunits without any homology (Figure 6A). Since the fourth amino acid pair from the NH_2 terminus was Pro/Lys, the Lys residue was blocked with *o*-phthalaldehyde, leaving the polypeptide chain with the Pro

Table III: Concentrations of Clusterin in Reproductive Tract Fluids and Sera of Rams

sample	clusterin (μL equiv/mL)	concn (μg/mL)	protein concn (mg/mL)	sp act. (μg of clusterin/mg of protein)
rete testis fluid (n = 25)[a]	1658 ± 785	161 ± 76	0.711 ± 0.23	239 ± 113
cauda epididymal fluid (n = 7)	4660 ± 170	452 ± 165	16 ± 1.3	29 ± 13
serum (n = 8)	540 ± 130	52 ± 12	36 ± 4.1	1.42 ± 0.3

[a] n denotes the number of rams used.

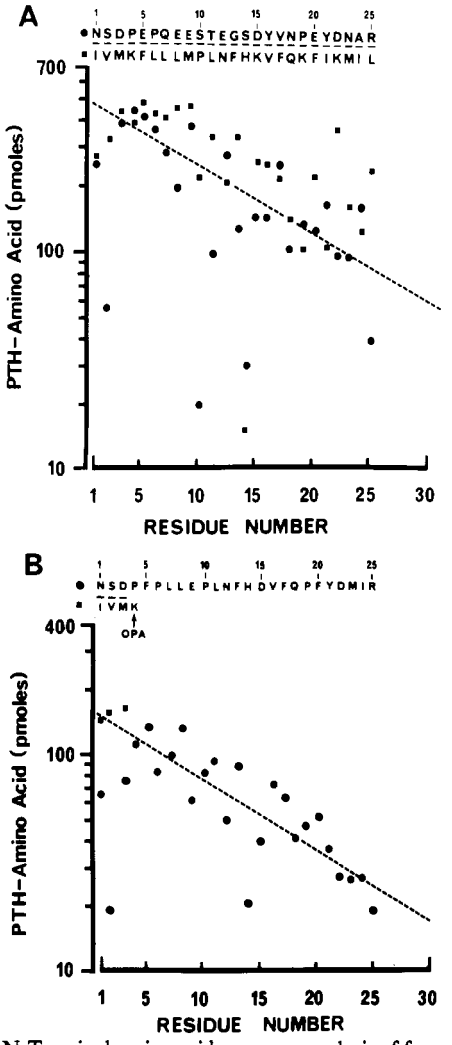

FIGURE 6: N-Terminal amino acid sequence analysis of form I clusterin (A) and its subunits using o-phthalaldehyde (B). Approximately 0.5 nmol of protein was sequenced by using the Applied Biosystem Inc. Model 470A protein sequencer. The repetitive yield was 95%, and the percentage blocking using o-phthalaldehyde was greater than 98%. Each sequence analysis was performed twice using two separate batches of samples, and identical results were obtained. Data shown here only represented the analysis of form I clusterin, since the N-terminal sequence analysis of form II clusterin yielded identical results as form I clusterin; thus, o-phthalaldehyde studies were only performed for form I clusterin.

residue accessible for subsequent Edman degradation. The amino acid sequence for this subunit from residues 4–25 was determined (Figure 6B). The amino acid sequences of the subunits of clusterin were compared to the release 10.0 of the protein sequence data base of the Protein Identification Resource (PIR) which consists of 4248 sequences containing 963 018 residues. Comparison of the amino acid sequences was done by using the optimizing algorithm (FASTP) of Lipman and Pearson (1985). No significant similarities were found between either one of the subunits of clusterin and the 4248 sequences in the data base. In addition, a comparison

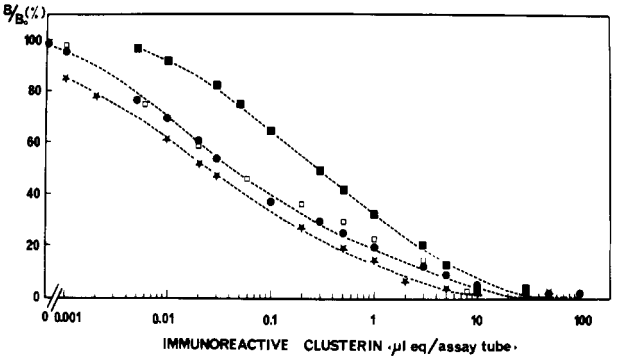

FIGURE 7: Competition of dilutions of serum (■), cauda epididymal fluid (★), rete testis fluid (●), and purified form I clusterin (□) with ^{125}I-clusterin for binding to monospecific polyclonal anti-clusterin antiserum. The abscissa is the log dose of competitor. The response is expressed as B/B_0 where B and B_0 are counts bound in the presence (B) and absence (B_0) of unlabeled competitor.

of the two subunits of clusterin failed to show any sequence homology. These analyses indicate that sequences 4–25 of the clusterin subunits are unique.

Distribution of Immunoreactive Clusterin in the Reproductive Tract and Serum. Serial dilutions of serum, rete testis fluid, cauda epididymal fluid, and purified form I clusterin were compared for their abilities to compete with radiolabeled clusterin for binding to the clusterin antiserum (Figure 7). Both serum, purified clusterin, and the reproductive tract fluids produced complete and parallel displacement of bound radioactivity. These results indicate that a protein in serum cross-reacts with testicular clusterin in the reproductive tract fluids. The concentrations of immunoreactive clusterin in the rete testis fluid, cauda epididymal fluid, and serum were determined and are shown in Table III. The concentration of clusterin is the highest in fluid from cauda epididymis, but the specific activity is the highest in rete testis fluid. The specific activity is 200- and 20-fold higher in the rete testis and cauda epididymal fluid, respectively, than in serum (Table III).

Partial Purification of Serum Clusterin and Its Biological and Physicochemical Characterization. Since serum clusterin cross-reacted with testicular clusterin, we thought it pertinent to determine whether these two proteins are of similar molecular size and that serum clusterin possesses similar biologic activity to the testicular protein. The serum clusterin was partially purified from a pool of ram plasma using an immunoaffinity column prepared from the IgG fractions of the clusterin antiserum. The scheme used for the partial purification of ram serum clusterin is outlined in Table IV. Immunoblots of the serum clusterin eluted from the immunoaffinity column had an apparent molecular weight of 37 000, slightly less than that of the testicular protein (Figure 8A, lanes 1 and 2). In the bioassay, serum clusterin did not aggregate erythrocytes in vitro, even when serum clusterin was present at concentrations 10-fold of that of the testicular clusterin (0.5 versus 0.05 μg of immunoreactive clusterin per assay well).

Biologic Activity of Deglycosylated Testicular Clusterin. Since immunoreactive serum clusterin did not have the in vitro biologic activity of the testicular protein and might be a

Table IV: Summary of the Purification Scheme for Immunoreactive Clusterin in Ram Plasma

step	[clusterin][a] (nmol)	[protein][b] (mg)	sp act.[c]	cumulative purification	recovery (%)
pooled ram plasma (52 mL)	28.9	4160	0.0069		100
immunoaffinity chromatography	0.29	1.02	0.2843	41.2	1

[a] The clusterin concentration was measured by radioimmunoassay using an apparent molecular weight of 80 000. [b] The protein concentration was determined by the dye binding assay with bovine serum albumin as a standard described under Experimental Procedures. [c] Specific activity was expressed as nanomoles of immunoreactive serum clusterin per milligram of protein.

nonglycosylated variant of the testicular protein, we sought to determine whether removal of the carbohydrate moiety from testiculr clusterin would affect its biologic activity. Purified testicular clusterin was deglycosylated by both enzymatic and chemical procedures. It was noted that both enzymatically and chemically deglycosylated testicular clusterin did not aggregate erythrocytes in vitro at concentrations 5–10-fold of that of the testicular clusterin required for the in vitro bioassay. It is of note that the removal of the carbohydrate moiety from the testicular clusterin by exoglycosidases reduced its apparent molecular weight on SDS–PAGE, making it similar to the serum clusterin (Figure 8A, lanes 4 and 5 versus lane 2). However, when testicular clusterin was deglycosylated using trifluoromethanesulfonic acid, there was a more dramatic change in the electrophoretic mobility on SDS–polyacrylamide gels than the enzymatically deglycosylated proteins (Figure 8B, lanes 1 and 2 versus lanes 3 and 4), corresponding to a change in apparent molecular weight of 5000 versus 3000, respectively. When these proteins were analyzed by lectin blots using WGA, a lectin specific for *N*-acetylglucosaminyl and sialic acid residues, it was of note that chemically deglycosylated clusterin lost its ability to interact with WGA (Figure 8B, lane 7) whereas enzymatically deglycosylated clusterin retained some WGA binding activity (Figure 8B, lane 6). It was also noted that the native clusterin migrated as a broad band on a lectin blot stained with WGA (Figure 8B, lane 5).

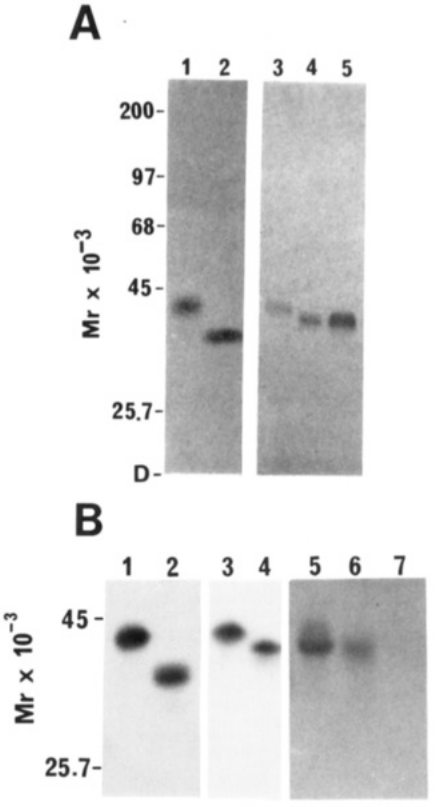

FIGURE 8: Analysis of clusterin from ram serum and rete testis fluid by immunoblots. (A) Serum clusterin was partially purified from a pool of serum by immunoaffinity chromatography (lane 2 consisting of about 50 ng of immunoreactive serum clusterin) and fractionated on a 10% *T* SDS–polyacrylamide gel along with ram testis fluid (lane 1 consisting of about 50 ng of immunoreactive clusterin in 0.5 μL of rete testis fluid sample). Enzymatically deglycosylated testicular clusterin (lanes 4 and 5 consisting of about 20 and 50 ng of immunoreactive form I clusterin, respectively) was fractionated with untreated clusterin (lane 3 consisting of 0.5 μL of ram rete testis fluid) on a 10% *T* SDS–polyacrylamide gel. Proteins in the SDS–polyacrylamide gels were transferred onto nitrocellulose paper and immunologically stained with the clusterin antiserum. (B) Relative mobilities of intact (lanes 1, 3, and 5), chemically deglycosylated (lanes 2 and 7), and enzymatically deglycosylated (lanes 4 and 6) ram testicular clusterin. Proteins (about 0.1 μg of purified clusterin per lane) were resolved on a 10% *T* SDS–polyacrylamide gel and transferred onto nitrocellulose paper. The paper was then divided: lanes 1–4 were immunologically stained with anti-ram clusterin antiserum; lanes 5–7 were stained with WGA.

Discussion

The present study confirms the observations of Fritz and co-workers (Fritz et al., 1983; Blaschuk et al., 1983) that clusterin is a glycoprotein which constitutes more than 10% of the total protein in the ram rete testis fluid and is a dimer consisting of two monomers of identical electrophoretic mobility on SDS–PAGE. The results of the present study show for the first time that the two subunits of clusterin consist of nonidentical N-terminal sequences. Whether there is any homology between the two subunits cannot be assigned until the entire sequence is known.

In the present study, we employed a somewhat different purification scheme than that previously reported (Blaschuk et al., 1983), and this leads to the identification of the two forms of clusterin. However, the cumulative yields for both purification schemes are comparable.

It is of note that clusterin is a glycoprotein reacting with WGA but not with concanavalin A. It is very likely that the carbohydrate constituents of the protein cause the differential interaction between the protein macromolecules and the Bio-Sil TSK 250 HPLC column matrix which is of a silica derivative (Hefti, 1982: Meredith, 1984; Josic et al., 1984). It is because of such an interaction that we derived two forms of clusterin from the fractionation. This conclusion has been reached on the basis of several lines of evidence: (1) identical N-terminal sequences for the two forms of clusterin; (2) virtually identical amino acid composition; (3) complete cross-reactivity as demonstrated by tandem crossed-immunoelectrophoresis; (4) virtually identical peptide maps except that they have a peptide fragment showing differential WGA affinity.

Development of a sensitive radioimmunoassay for clusterin has permitted us to investigate the distribution of clusterin in the reproductive tract fluids and the serum. The differential concentrations and specific activities of clusterin in the reproductive tract fluids are similar to those of other Sertoli cell proteins that are secreted into the tubular fluid and transported to the epididymis via the rete testis [for reviews, see Bardin et al. (1981, 1988) and Hansson et al. (1975)]. It is of interest to note that the specific activities of clusterin (micrograms of clusterin per milligram of protein) in the rete testis fluid and cauda epididymal fluid are 240 and 30, respectively, versus 1.4 in the serum. Thus, the concentration of clusterin is several orders of magnitude larger in the reproductive tract fluid than

in the serum. It is of note that the levels of clusterin in the cauda epididymal fluid and serum of the ram reported in this paper (450 and 50 μg/mL, respectively) are somewhat higher than those reported earlier (Tung & Fritz, 1985). These authors reported the concentrations of clusterin in these fluids using an enzyme-linked immunoassay are 80–200 and 0.8–3.2 μg/mL, respectively. The discrepancy between these reported values is not immediately known. These differences might be attributed to the different breeding status of the animals subjected to studies because we have recently observed that the levels of clusterin in the reproductive tract fluids vary dramatically with the breeding cycle of the ram (Mathur, Bardin, Voglmayr, and Cheng, unpublished observations).

The demonstration of immunoreactive clusterin in the vascular compartment has raised some interesting questions with regard to the in vivo biological function of this protein. In vitro biological assays using erythrocytes have shown that it requires as little as 0.05 μg of clusterin to elicit cell aggregation and that the concentration of immunoreactive clusterin in the serum is about 50 μg/mL, which is sufficient enough to induce cell aggregation. Since clusterin causes aggregation of erythrocytes, therefore, one would expect this protein to be confined within the testicular and/or the epididymial compartments. Thus, the presence of this protein in the vascular compartment seems to suggest an alternative physiologic function(s) for this protein rather than cell aggregation unless there is a potent clusterin inhibitor present in the serum. We have validated the presence of this protein in the vascular compartment by both radioimmunoassay and immunoblot using partially purified serum clusterin obtained by immunoaffinity chromatography. Both approaches indicate that clusterin is indeed present in the serum. Serum clusterin has an apparent molecular weight of 37 000 and is likely to be a deglycosylated form of clusterin. Subsequent in vitro bioassays using immunoreactive serum clusterin at a concentration equivalent to the testicular clusterin required for the in vitro bioassay or 10-fold of that concentration indicated that the serum clusterin does not possess the biologic activity of the testicular protein to elicit cell aggregation. These observations are consistent with the postulate that the serum protein has undergone posttranslational processing which renders it to be inactive in the bioassay. Deglycosylation of testicular clusterin has also rendered the protein to be inactive to elicit cell aggregation, providing further evidence that the carbohydrate core of clusterin is important for cell aggregation.

The origin of clusterin in the serum is not known. Immunohistochemical localization of clusterin in the reproductive tract indicates that the Sertoli cell is the possible origin of this protein in the testicular compartment (Tung & Fritz, 1985). However, extragondal site(s) of synthesis for this protein possibly exist(s); recent studies using immunoprecipitation have shown that the epithelial cells of the rete testis cultured in vitro can synthesize and secrete immunoreactive clusterin into the medium (Rosenior et al., 1987). Such a phenomenon is not entirely unusual. Recent evidence suggests that the Sertoli cell is the "hepatocytic" counterpart of the testis in which it synthesizes and secretes a number of liver proteins including transferrin, ceruloplasmin, retinol binding protein, and growth factor (Skinner & Griswold, 1980, 1983; Skinner et al., 1984; Carson et al., 1984; Holmes et al., 1986). In some instances, the Sertoli cell is producing a protein very similar to the liver but not identical. An example of this is the androgen binding protein in which this protein shares almost identical features with the serum sex hormone binding globulin both structurally, immunologically, and physiologically (Musto et al., 1982; Joseph et al., 1987; Bardin et al., 1988). However, whether serum clusterin is originated from the liver or is being synthesized by other organs needs further investigation.

Thus, we have purified clusterin from the ram rete testis fluid and have determined the N-terminal sequences for each of the subunits of clusterin. We have also validated a highly sensitive radioimmunoassay for clusterin and have measured the distribution of this protein in the reproductive tract fluids. In addition, we have shown that clusterin is not restricted within the testicular compartment and is present in significant quantity in the serum. Serum clusterin appears to be a nonglycosylated variant of the testicular protein, but it lacks the bioactivity of testicular clusterin in vitro. Removal of the carbohydrate moiety from testicular clusterin would also render this protein to be inactive in the in vitro bioassay. Work is now in progress to understand the structural relationship of the serum and testicular clusterin and to determine the physiologic role of clusterin in the serum.

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