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RAT CLUSTERIN ISOLATED FROM PRIMARY SERTOLI CELL-ENRICHED CULTURE MEDIUM IS SULFATED GLYCOPROTEIN-2 (SGP-2)

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SUMMARY: Clusterin, a glycoprotein originally isolated from ram rete testis fluid, is a dimer composed of monomers with non-identical NH_2-terminal amino acid sequences. In view of its possible role in cell-cell interactions in the seminiferous epithelium, we sought to identify such a protein in the rat. Using the bioassay developed for the ovine protein, rat clusterin was purified to apparent homogeneity by HPLC from primary Sertoli cell-enriched culture media. This protein is also a heterodimer consisting of monomers of Mr 43,000 (α) and Mr 35,000 (ß). NH_2-Terminal amino acid sequence analysis indicated that the α subunit has a sequence of NH_2-SLMPLSHYGPLSFHNMFQPFFDMIHQAQQA and the ß subunit, NH_2-EQEFSDNELQELSTQGSRYVNKEIQNAVQG. These two subunits show marked similarity with the corresponding subunits of ram clusterin isolated from rete testis fluid. Using an antibody against the α subunit of rat clusterin, a cDNA clone was isolated from a rat testicular lambda gt11 cDNA library. Analyses of the amino acid sequence derived from the isolated rat clusterin cDNA and of the NH_2-terminal amino acid sequences indicate that rat clusterin is identical to a Sertoli cell glycoprotein previously designated sulfated glycoprotein-2.

Clusterin is a glycoprotein of Mr 80,000 present in the rete testis fluid of the ram that elicits aggregations of Sertoli cells and erythrocytes from several species in vitro (1,2). Immunohistochemical studies revealed that clusterin is localized in the ram Sertoli cells of the seminiferous tubules (3), and epithelial cells of rete testis and epididymis (4). NH_2-terminal amino acid sequence analysis indicated that ram clusterin is a heterodimer (5). The apparent molecular weights of the individual monomers of ram clusterin are 40,000 (2, 5); however, in some batches of rete testis fluids, the two monomers displayed molecular weights of 40,000 and 37,000, respectively (4, 6). Since this protein elicits cell aggregations in vitro, it was postulated that this activity might be important for cell-cell interactions in the seminiferous epithelium (1, 3-6). Thus, we sought to purify and characterize rat clusterin from primary Sertoli cell-enriched culture medium.

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MATERIALS AND METHODS

Preparation of primary Sertoli cell-enriched culture medium. Sertoli cell-enriched cultures were prepared from 20-day-old Sprague-Dawley rats as previously described (7), and media were prepared for HPLC fractionations as detailed elsewhere (8-10).

In vitro bioassay of clusterin. The column fractions obtained from each purification step and the purified proteins were assayed for clusterin activity using erythrocytes prepared from adult Sprague-Dawley rats (5).

Purification of rat clusterin. *Anion-exchange HPLC*: A 4 liter pool of primary Sertoli cell-enriched culture medium was concentrated and equilibrated in buffer A (20 mM Tris, pH 7.4, at 22C) and prepared for anion-exchange HPLC fractionation as previously described (10) using a preparative Mono Q anion-exchange HPLC column (HR 10/10, 10 x 100 mm i.d.) at a flow rate of 4 ml/min. *Hydroxylapatite HPLC*: Fractions containing rat clusterin were pooled and equilibrated against solvent A (10 mM sodium phosphate, 0.3 mM $CaCl_2$, pH 7, at 22C) and loaded onto a hydroxylapatite HPLC column [Bio-Gel HPHT column (7.8 x 100 mm i.d.) equipped with a Bio-Gel HPHT guard column (4 x 50 mm i.d.)] at a flow rate of 1 ml/min using procedures as previously described (9). Proteins were eluted using a linear gradient of 0-80% solvent B (300 mM sodium phosphate, 0.01 mM $CaCl_2$, pH 7, at 22C) over a period of 30 min. Fractions containing rat clusterin were pooled and lyophilized. *Phenyl-reverse phase HPLC*: The lyophilized protein was resuspended in solvent A (5% acetonitrile/95% H_2O, V/V containing 0.1% TFA) and loaded onto a Vydac phenyl-reverse phase HPLC (Model 219TP54, 4.6 x 250 mm i.d., particle size 5 μm) using procedures essentially as previously described (11). Proteins were eluted using a linear 20-70% solvent B (95% acetonitrile/5% H_2O, V/V containing 0.1% TFA) over a period of 45 min at a flow rate of 1 ml/min.

NH_2-Terminal sequence analysis of rat clusterin. Edman degradation of the native protein using the Applied Biosystem Inc. Model 470A gas-phase protein sequencer using procedures as previously described (5) yielded two amino acids in each cycle consistent with the observations that this protein is a heterodimer. We have thus sequenced each subunit using electroeluted proteins from SDS-polyacrylamide gels. Briefly, purified rat clusterin (about 5 μg/lane) was resolved by SDS-PAGE under reducing conditions. Following electrophoresis, gel slices corresponding to each of the two subunits were removed and transferred to a dialysis membrane (Mr cut-off at 6,000-8000) containing 5 ml of 0.02M Tris, 0.2M glycine, pH 7.4, at 22C, and was suspended in the same buffer in a Hoefer Minnie-submarine gel unit (Model HE33). Electrophoresis was performed at constant voltage (60V) for at least 12 h at room temperature. Electroeluted protein was withdrawn from the dialysis membrane and equilibrated extensively against double distilled water on an Amicon Centricon-10 microconcentrator and resuspended in 0.1% SDS solution before Edman degradation. About 0.1-0.2 nmol of each subunit was sequenced.

cDNA-cloning and nucleotide sequencing. The rat testicular cDNA library constructed in lambda gt11 used in this study was a gift of Dr. Anthony R. Means at Baylor College of Medicine (13). This library was screened with a monospecific antiserum prepared in a rabbit against the α-subunit of rat clusterin using protein A-peroxidase (14). The clone containing the longest cDNA coding for rat clusterin, RT102, was further characterized by epitope selection (15). It was then subcloned into pGEM™-3 blue vector (Promega Biotec, Madison, WI) designated pRT102. Nucleotide sequences from both orientations were analyzed by dideoxynucleotide chain termination method using Sequenase™ obtained from United States Biochemical Corp. (Cleveland, OH) (16).

Northern blot analysis. Total RNA was isolated from rat and ram testes using a urea-lithium chloride precipitation procedures (17) and poly(A)-containing RNA was obtained by oligo(dT)-cellulose chromatography (18). Poly(A)-RNA was denatured, fractionated on agarose gel, and transferred to GeneScreen Plus™ hybridization membrane (Du Pont) as previously described (19, 20). The clusterin mRNA was identified by hybridization with ^{32}P-labeled rat clusterin cDNA (pRT102).

RESULTS

Purification of Rat Clusterin. From approximately 4 liters of Sertoli cell-enriched culture medium which has a total protein content of about 130 mg, 250 μg of protein with clusterin bioactivity was obtained using three sequential HPLC steps involving

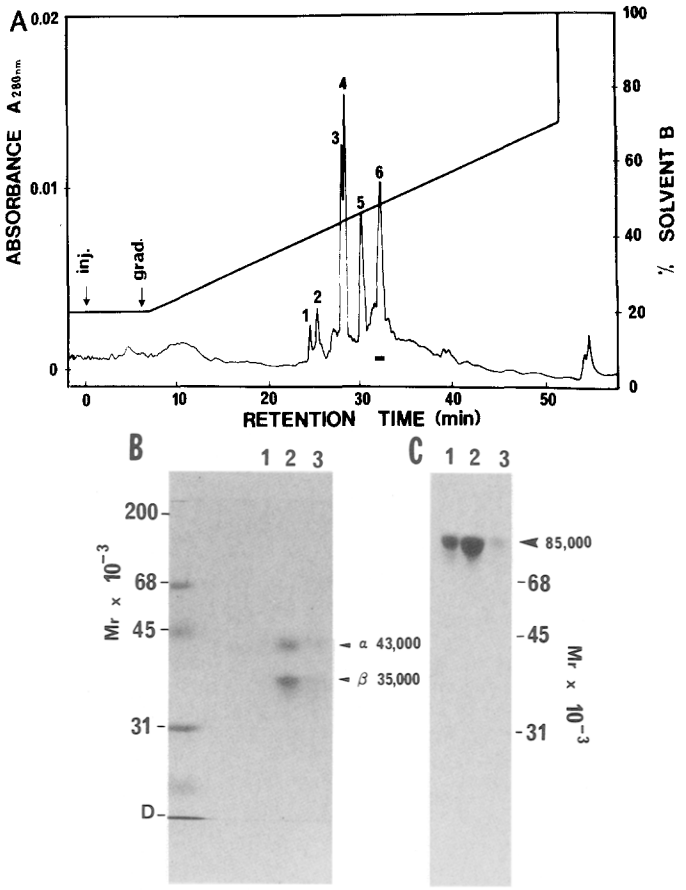

Fig. 1A, B, C. Fractionation of rat clusterin by phenyl-reverse phase HPLC and SDS-PAGE. **(A).** Purification of rat clusterin by phenyl-reverse phase HPLC. Partially purified rat clusterin obtained by sequential anion-exchange and hydroxylapatite HPLC was lyophilized and resuspended in solvent A (5% acetonitrile/95% H_2O, V/V containing 0.1% TFA) and loaded onto a Vydac 219TP54 phenyl-reverse phase HPLC column. Proteins were eluted using a gradient of 20-70% solvent B (95% acetonitrile/5% H_2O, V/V containing 0.1% TFA) over a period of 45 min at a flow rate of 1 ml/min. A total of 6 protein peaks were identified and rat clusterin bioactivity was eluted under peak 6 as indicated by the bar. Inj. indicates where sample was loaded; grad. indicates where elution of proteins was started. **(B), (C).** SDS-polyacrylamide gel electrophoresis of purified rat clusterin under reducing (B) and non-reducing conditions (C) in the presence and absence of 2-mercaptoethanol, respectively. In both panels, lanes 1-3 corresponded to the fractions under peak 6 shown in Fig. 1A in which 0.5, 3, and 1 μg of proteins were resolved onto the gel, respectively. Gels were stained with Coomassie blue because rat clusterin could not be readily stained with silver nitrate. Arrows indicate the two subunits of rat clusterin.

anion-exchange, hydroxylapatite, and phenyl-reverse phase columns. The elution profile of the phenyl-reverse phase HPLC is shown in Fig. 1A; clusterin activity is located in fractions under peak 6. Six batches of this protein have been isolated with an overall recovery of about 18%. The purified clusterin when resolved by SDS-PAGE under reducing conditions was composed of two subunits of α and ß with apparent molecular weights of 43,000 and 35,000, respectively (Fig. 1B). However, when this protein was

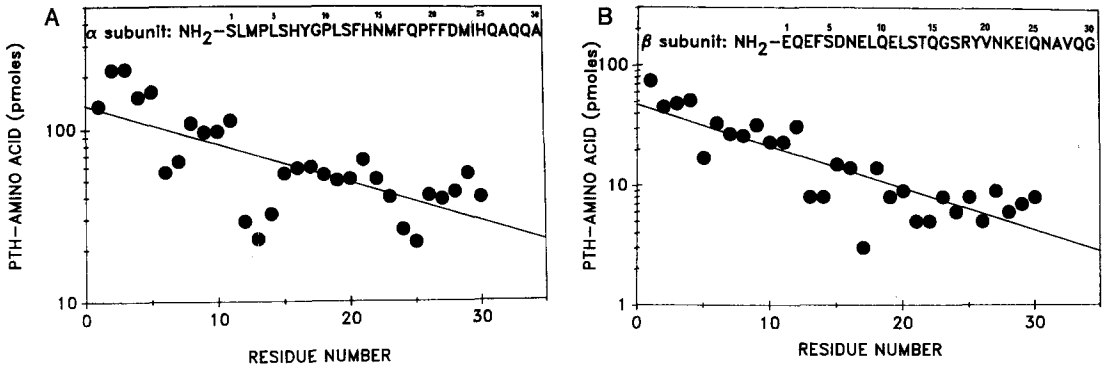

Fig. 2 A, B. NH_2-Terminal sequence analysis of the α (A) and ß subunits (B) of rat clusterin. Approximately 200 pmol of the α subunit and 100 pmol of the ß subunit were sequenced using the Applied Biosystem Inc. Model 470A gas-phase sequencer. The released phenylthiohydantoin-amino acids (PTH-amino acids) were analyzed by reverse-phase HPLC using a Hewlett-Packard liquid chromatography terminal (Model 1084A) and a Du Pont Zorbax ODS HPLC column. The repetitive yield was 95%.

fractionated by SDS-PAGE under non-reducing conditions (Fig. 1C), it displayed a single band of Mr 85,000. We conclude from these data that rat clusterin is a heterodimer of Mr 85,000 consisting of monomers of Mr 43,000 and Mr 35,000 that are linked by disulfide bond(s). In the in vitro bioassay, as little as 0.1 μg of purified rat clusterin was needed to elicit cell aggregation in a 0.1 ml suspension of erythrocytes.

NH_2-Terminal Sequence Analysis of Rat Clusterin. Microsequence analysis of the first 30 amino acids from the NH_2-termini of the rat clusterin subunits are shown in Fig. 2 A, B. Comparison with the NH_2-terminal sequences of ram clusterin (5) revealed a marked similarity between these proteins (Fig. 3). Unexpectedly, the NH_2-terminal sequences of the two subunits of rat clusterin were identical to that of SGP-2 (Fig. 3) (28).

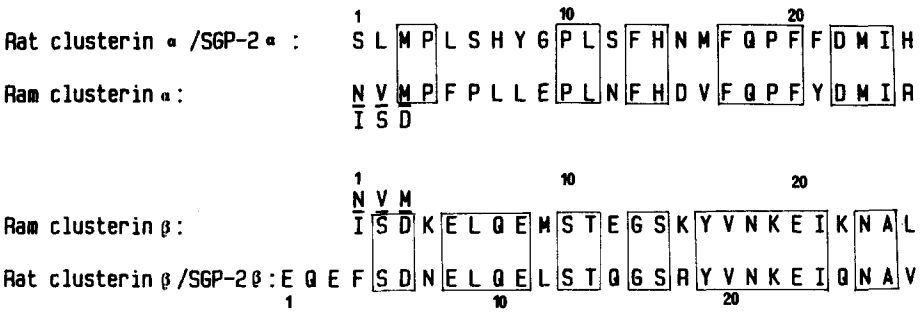

Fig. 3. Comparison of the NH_2-terminal amino acid sequence of the two subunits of rat and ram clusterin, and SGP-2. Data for the NH_2-terminal sequence of the two subunits of ram clusterin and SGP-2 were derived from Cheng et al. (5) and Collard and Griswold (28), respectively.

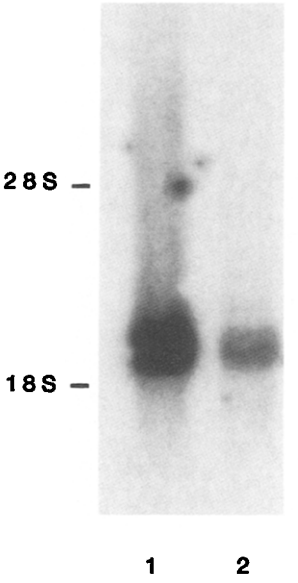

Fig. 4. Identification of testicular clusterin mRNA. Ten μg each of testicular poly(A)RNA isolated from rat (lane 1) and ram (lane 2) were subjected to Northern blot analysis, and clusterin mRNA was identified using isolated rat clusterin cDNA (pRT102) as a hybridization probe.

Isolation and Sequencing of cDNA Coding for Rat Clusterin. Rat testicular cDNA library constructed in lambda gt11 was screened with a monospecific antibody prepared against the α-subunit of rat clusterin. Three positive clones were isolated from $5x10^5$ phage plaques. The clone containing the longest cDNA insert, RT102, was further identified by epitope selection (15). The nucleotide sequence of this clone showed that it was 783 bp in length and corresponded to nucleotides in SGP-2 cDNA between position 812 through the entire 3'-untranslated region to poly(A) tail (28). The nucleotide sequence of pRT102 is identical to that reported for SGP-2 cDNAs except that 5 additional nucleotides (GCGAT) were found preceding the poly(A) tail at the 3' end.

Identification of the mRNA for Rat Clusterin. The mRNA coding for rat testicular clusterin was identified by Northern blot analysis (Fig. 4) using isolated pRT102 as hybridization probe. A single mRNA species of approximately 2 Kb was identified in rat testis. A similar result with weaker intensity was obtained in ram testis, suggesting that ram clusterin mRNA shares sequence homology with that of the rat.

DISCUSSION

Previous studies of ram clusterin showed that its monomers have dissimilar NH_2-terminal amino acid sequences (5). Since this protein appears to be physiologically important in view of its high concentration in the reproductive tract fluids and its in vitro biological activity, we isolated this protein from the rat primary Sertoli cell-enriched culture medium. The rat protein with clusterin activity is also a heterodimer with two subunits of Mr 43,000 and 35,000, respectively. In contrast, the subunits of the ram clusterin have Mr 40,000 in most preparations. However, size heterogeneity of the ß subunit was observed in the ram rete testis fluid in that some preparations had a subunit of Mr 37,000 (4, 6). In the present study, we demonstrated structural similarity of clusterins from these two species as shown by their NH_2-terminal amino acid sequences.

Several laboratories have recently identified a number of Sertoli cell proteins other than androgen binding protein including growth factors (21, 22), transferrin (23), ceruloplasmin (24), retinol-binding protein (25), CP-2 (26), SGP-2 (27, 28), and others (8-10, 29). In an attempt to isolate another rat Sertoli cell product, a protein was isolated using the clusterin bioassay that proved to be SGP-2. The identity of clusterin and SGP-2 is based upon NH_2-terminal sequence analysis of the rat proteins, the predicted amino acid sequence of a cDNA selected with antibodies against rat clusterin, and the similarity of the NH_2-terminal sequence of rat clusterin/SGP-2 and ram clusterin.

The original studies of clusterin in ram by Fritz and his collaborators (1-4) demonstrated that this protein could induce cell adhesion. These results suggested that this protein could facilitate cell-cell interactions in the reproductive tract. Subsequent experiments demonstrated that this protein is present in the vascular compartment and that serum clusterin does not have the cell aggregating activity (5). Furthermore, deglycosylated ram testicular clusterin does not aggregate cells in vitro (5). These latter studies suggest that the function(s) of this protein in the reproductive tract and vascular compartment may be different provided that the in vitro bioactivity correlates with the in vivo activity. The amino acid sequence of rat SGP-2 as predicted from the cDNA sequence showed that there is similarity with members of the apolipoprotein family,

these observations suggested to Griswold and collaborators that this protein may be involved in lipid transport (28). Since it is now clear that clusterin and SGP-2 are the same protein and that they are present in multiple tissues and species, it will be important to determine whether this protein has one or multiple function in vivo.

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