

Germ Cell-Conditioned Medium Contains Multiple Factors that Modulate the Secretion of Testins, Clusterin, and Transferrin by Sertoli Cells

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ABSTRACT: In view of the evidence showing that germ cells regulate Sertoli cell (SC) function, the aim of this study was to examine if germ cell (GC)-conditioned media contained multiple biological factors that affect SC secretory functions. Total GC were isolated from adult rat testes. Pachytene spermatocytes (SPC) and early spermatids (SPT) were enriched to about 90% pure by centrifugal elutriation. GC, SPC, and SPT were cultured in serum-free medium for 20 hours with a viability greater than 95%. Conditioned media derived from these cells were fractionated by anion-exchange high-performance liquid chromatography (HPLC). An aliquot from each of these fractions was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and proteins were visualized by silver staining. The patterns of protein staining using media from GC, SPC, and SPT were similar. Bioassays of these column fractions on SC showed that the transferrin stimulatory, the testins inhibitory, and the clusterin inhibitory activities were eluted from the anion-exchange HPLC column in overlapping fractions. To determine whether these activities were confined to one or several molecules, further fractionations were performed. Eleven liters of GC-conditioned medium were fractionated by sequential HPLC using anion-exchange, gel permeation,

and reversed-phase columns. Throughout the entire fractionation scheme, the HPLC fractions were bioassayed using primary SC-enriched cultures prepared from 20-day-old rats by incubating SC with aliquots of these fractions for 24 hours to monitor their effects on SC secretory function. The concentrations of transferrin, clusterin, and testins were quantified by specific radioimmunoassays. These studies showed that the transferrin stimulatory activity can be fractionated into four peaks (I, IIa, IIb, and IIc); clusterin inhibitory activity into three peaks (A, B, and C); and testins inhibitory activity into two peaks (1 and 2). Some of these bioactivities were eluted in overlapping fractions such as I and B, IIb and 1, and IIc and 2, whereas A, C, and IIa were not associated with any other assayed activities. In summary, additional GC modulators of SC function were identified for the first time, these include clusterin and testins inhibitors. The previously identified transferrin stimulatory activity was also resolved into multiple molecular forms.

Key words: HPLC, early spermatid, pachytene spermatocyte, transferrin, clusterin, testins.

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Sertoli cells (SC) are a major somatic component in the seminiferous epithelium and are known to synthesize and secrete many serum and testis-specific proteins that in turn determine the fluid composition in the seminiferous tubular lumen (Bardin et al, 1988; Cheng et al, 1991). Based on the intimacy between SC and germ cells (GC) in the seminiferous epithelium, it has been postulated that SC plays a major role in regulating GC development. Several SC markers are now available to probe these events including transferrin, androgen binding protein (ABP), inhibin, clusterin (sulfated glycoprotein-2), α_2 -macroglobulin, and testins (for reviews, see Bardin et al, 1988; Cheng et al, 1991).

Other studies have shown that GC synthesize proteins at almost every stage of spermatogenesis particularly at the pachytene stage of meiosis (Monesi, 1967). Some GC proteins are synthesized transitory (Meistrich et al, 1981), whereas others fluctuate during spermatogenesis (Parvi-

nen, 1982). Numerous *in vitro* studies strongly suggest that GC release biological factors that modulate SC secretory function. For example, GC-conditioned medium was found to stimulate the phosphorylation of specific SC proteins (Ireland and Welsh, 1987); increase ABP, transferrin, and inhibin secretions; and decrease estradiol productions (Le Magueresse and Jégou, 1986, 1988a; Djakiew and Dym, 1988; Le Magueresse et al, 1988; Pineau et al, 1990). Also, it has been shown that GC-conditioned medium affects the mRNA levels of both transferrin (Stallard and Griswold, 1990) and the α -subunit of inhibin (Pineau et al, 1990).

In the present study we have attempted to fractionate GC-conditioned medium to determine if it contains multiple biological factors that affect the secretion of clusterin, testins, and transferrin by SC. These are the subjects of this report.

Materials and Methods

Biochemicals

Serum-free culture medium was prepared by mixing Dulbecco's modified Eagle's medium (DMEM) and nutrient mixture Ham's

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F12 in a ratio of 1:1 (v/v) supplemented with sodium bicarbonate (1.2 g/L), HEPES (15 mM), and gentamycin (20 mg/L). Acrylamide (40%, w/v) was obtained from International Biotechnologies, Inc. (New Haven, CT). Glycine, Tris, sodium dodecyl sulfate (SDS), ammonium persulfate, *N,N'*-methylenebis(acrylamide), TEMED (*N,N,N',N'*-tetramethylethylenediamine), DATD (*N,N'*-diallyltartardiamide), 2-mercaptoethanol, and high and low molecular weight standards were from Bio-Rad (Richmond, CA). Silver nitrate and citric acid were from Aldrich Chemical Co. (Milwaukee, WI). Methanol and formaldehyde solution (37%, v/v) were from Fisher Scientific Co. (Fair Lawn, NJ). [¹²⁵I]-Bolton-Hunter reagent [*N*-succinimidyl 3-(4-hydroxy 5-[¹²⁵I]iodophenyl propionate; specific activity, 3,024–3,320 Ci/mmol] was obtained from ICN Radiochemicals (Irvine, CA). Immunoprecipitin (formalin-fixed *Staphylococcus aureus* cells) were obtained from BRL (Gaithersburg, MD). Bovine serum albumin (BSA, fraction V), trypsin, collagenase, hyaluronidase, soybean trypsin inhibitor, bacitracin, DNase, sodium lactate, and sodium pyruvate were obtained from Sigma (St. Louis, MO).

Preparation of GC-Conditioned Medium

GC were prepared from adult Sprague-Dawley rat testes (Elevage Janvier, Le Genest Saint Isle, France) by trypsin digestion as previously described (Meistrich et al, 1981). Enriched populations of pachytene spermatocytes (SPC) and early spermatids (SPT) were prepared by centrifugal elutriation with a purity of greater than 90% (Meistrich et al, 1981). GC, SPC, and SPT were incubated individually at 32°C for 20 hours in Ham's F12/DMEM (v/v) (Gibco BRL, France) supplemented with 2 mM sodium pyruvate and 6 mM sodium DL-lactate at a density of 2.5×10^6 , 2.5×10^6 , and 8×10^6 cells/ml, respectively, in 175-cm² tissue culture flasks (Nunc, Copenhagen, Denmark). After 20 hours, GC and cellular debris were removed by successive centrifugations ($2 \times 200 \times g$ at 4°C for 10 minutes followed by $10,000 \times g$ at 4°C for 1 hour). Preliminary experiments showed that 95% of GC were viable at this time as judged by the erythrocyte red dye exclusion test. Concentration and equilibration of GC-conditioned medium were performed at 4°C using a Millipore Minitan[®] tangential ultrafiltration unit equipped with eight Minitan[®] plates with an Mr cutoff at 10,000.

DNA Flow Cytometry

The relative DNA content of cells in suspension was estimated by DNA flow cytometry as previously described (Jutte et al, 1982). Briefly, the cells were treated with 0.4% pepsin in 0.02 M HCl at 37°C for 15 minutes and then incubated for 30 minutes at room temperature in a mixture of ribonuclease-A (10 mg/ml), ethidium bromide (10 mg/ml), and Nonidet P-40 (300 µl/L). The samples were filtered through a 5.5-µm nylon mesh and analyzed using a flow cytometer as previously described (Le Magueresse and Jégou, 1988b). The proportions of cells with a haploid (1C), diploid (2C), and tetraploid (4C) DNA content were calculated by measuring the areas under the corresponding peaks.

Fractionation of Proteins in GC-Conditioned Media

Anion-Exchange High-Performance Liquid Chromatography (HPLC)—GC-, SPT-, and SPC-conditioned media were con-

centrated and equilibrated individually against solvent A (20 mM Tris, pH 7.4, at 22°C) and loaded onto an anion-exchange HPLC analytical column (Mono Q, HR 5/5, 5 × 50 mm, i.d.; particle size, 10 µm) at a flow rate of 1 ml/minute as previously described (Cheng et al, 1986, 1989). Bound proteins were eluted using a linear gradient of 0–80% solvent B (20 mM Tris, pH 7.4 at 22°C, containing 600 mM NaCl) at a flow rate of 1 ml/minute for 45 minutes. Fractions (1 ml each) were collected and the eluents were monitored by UV absorbance at 280 nm. An aliquot from each fraction was then resolved by SDS-polyacrylamide gel electrophoresis (PAGE) onto 10% T SDS-polyacrylamide gels, and proteins were visualized by silver nitrate. Preparative fractionation was performed using 11 L GC-conditioned media using a Mono Q preparative column (HR 10/10, 10 × 100 mm, i.d.; particle size, 10 µm) as previously described (Cheng and Bardin, 1986). The fractionation procedures were the same as the analytical column except that a flow rate of 3 ml/minute was used.

Gel Permeation HPLC—Following the preparative anion-exchange HPLC, the biologically active fractions were pooled and concentrated to about 100 µl by sequential ultrafiltration using a YM-10 membrane on an Amicon ultrafiltration unit (model 8010) and an Amicon Centricron-10 microconcentrator (Amicon, Danvers, MA). It was then loaded onto a Pharmacia Superose 12 gel permeation HPLC column (10 × 300 mm, i.d.). Proteins were eluted under isocratic conditions using 10 mM sodium phosphate, pH 6.8, at 22°C, containing 0.15 mM NaCl, at a flow rate of 0.8 ml/minute. Fractions (0.8 ml each) were collected and eluents were monitored by UV absorbance at 280 nm. An aliquot from each fraction was then resolved by SDS-PAGE on 12.5% T gels, and proteins were visualized by silver nitrate.

Reversed-Phase HPLC—Following the preparative anion-exchange HPLC step, fractions containing the biological activities were pooled, lyophilized, and resuspended in 500 µl of solvent A [5% acetonitrile (ACN)/95% H₂O, containing 0.1% trifluoroacetic acid (TFA), v/v] and loaded onto a Vydac C8 reversed-phase HPLC column (4.6 × 250 mm, i.d.; particle size, 10 µm) at a flow rate of 1 ml/minute. Bound proteins were eluted with a linear gradient of 5–80% solvent B [95% ACN/5% H₂O, containing 0.1% TFA, v/v] over a period of 60 minutes at a flow rate of 1 ml/minute. Eluents were monitored by UV absorbance at 280 nm. Fractions of 1 ml each were collected. An aliquot from each fraction was withdrawn and fractionated by SDS-PAGE onto 12.5% T SDS-polyacrylamide gels, and proteins were visualized by silver nitrate.

Preparation of SC-Enriched Cultures

SC were prepared from testes of 20-day-old Sprague-Dawley rats (Charles River Laboratories, Kingston, MA) as previously described (Skinner and Fritz, 1985). The SC-enriched suspensions were seeded at a density of approximately 2×10^5 cells per well in 24-well culture dishes (Falcon, Becton Dickinson, Lincoln Park, NJ). The cells were then incubated at 35°C in a humidified atmosphere of 5% CO₂ and 95% air (day 0 of culture) in 500 µl Ham's F12/DMEM (v/v) (Gibco BRL, Gaithersburg, MD) supplemented with insulin (10 µg/ml), human transferrin (5 µg/ml), epidermal growth factor (2.5 ng/ml), and bacitracin (10 µg/ml).

Culture media were replenished daily until the end of the experiment. On day 2 of the culture, SC were exposed to a 20-mM Tris-HCl buffer solution (pH 7.4) for 2.5 minutes to remove contaminating GC (Galdieri et al, 1981). These cells were then used for bioassay on day 3.

Bioassay of GC Factors that Modulate SC Secretory Functions

Aliquots of column fractions (between 2 and 50 μ l) were added to 24-well dishes containing SC and incubated for 24 hours. Each column fraction was bioassayed at two doses using triplicate wells. Thereafter, spent media were collected, and SC proteins in each culture well were quantified by corresponding specific radioimmunoassays. A laboratory standard was established using a pool of total GC-conditioned media designated CPLRS1, which was run in every bioassay at volumes equivalent to aliquots of column fractions. Negative controls were prepared using HPLC buffers. Control incubations represented culture wells containing SC alone without added column fractions.

Analytical PAGE

Analytical PAGE in the presence of SDS was performed as previously described (Laemmli, 1970) and modified in this laboratory (Cheng et al, 1983). The resolving gels consisted of 10%, 12.5%, or 14% *T* [total gel concentration (g/100 ml) = acrylamide + methylene-bisacrylamide] and 2.6% cross-linker using methylene-bisacrylamide (% C_{Bis}) with stacking gel of 5% *T* and 15% *N,N'*-diallyltartardiamide (% C_{DATD}). Throughout the entire fractionation, an aliquot from each HPLC fraction was denatured in SDS sample buffer (0.125 M Tris, pH 6.8, at 22°C containing 1% SDS, 1.6% 2-mercaptoethanol, and 10% glycerol) at 100°C for 5 minutes. Gels were routinely stained with silver nitrate (Wray et al, 1981).

General Methods

Protein estimation was performed by Coomassie blue-dye binding assay (Bradford, 1976) as modified elsewhere (Macart and Gerbaut, 1982). Rat transferrin, clusterin, and testins were quantified by specific radioimmunoassays as previously described (Rossi et al, 1989; Grima et al, 1990; Cheng and Bardin 1987; Cheng et al, 1989).

Results

Purity of the GC Preparations

Total GC isolated by trypsinization are shown in Figure 1A. A representative pattern of DNA fluorescence of such a preparation is presented in the inset of Figure 1A. Haploid nuclei (1C) predominated; diploid (2C) and tetraploid nuclei (4C) were less prominent. SPT (Fig. 1B) and SPC (Fig. 1C) were enriched by subsequent centrifugal elutriation to a purity of at least 90%. Inserts in Figure 1B,C illustrate the patterns of DNA fluorescence of the SPT (1C) and the SPC (4C) fractions, respectively. In these preparations, the 2C peaks were composed of spermatids with two nuclei, spermatogonia, and SC. The viability of

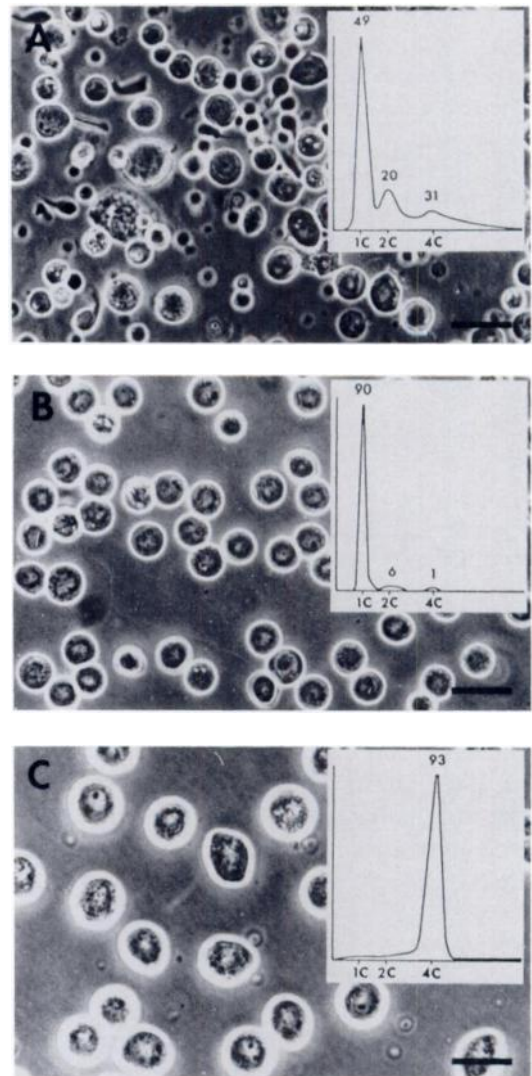


FIG. 1. Photomicrographs of total germ cells (A), highly purified early spermatids (B), and pachytene spermatocytes (C) isolated from adult rat testis by trypsinization and centrifugal elutriation as described in *Materials and Methods*. Bar: 25 μ m. The insets show the respective DNA distribution pattern. The proportions of cells with a haploid (1C), diploid (2C), and tetraploid (4C) DNA content are noted above each peak. x-axis: channel (relative fluorescence intensity); y-axis: cells/channel (relative units).

these cell preparations was greater than 95% when assessed by the erythrosine red exclusion test. More importantly, these cells still maintained a viability of greater than 90% at the end of the 20 hours of incubation period. However, incubation of GC for over 24 hours caused a dramatic decrease in viability (Le Magueresse and Jégou, 1988a). The total protein contents in the GC-, SPT-, and SPC-conditioned media were 5, 30, and 10 μ g/ml when these cells were cultured at a density of 2.5×10^6 , 8×10^6 , and 2.5×10^6 cells/ml, respectively. It is not unexpected that conditioned medium derived from a mixed population of GC contains less proteins than SPT- and

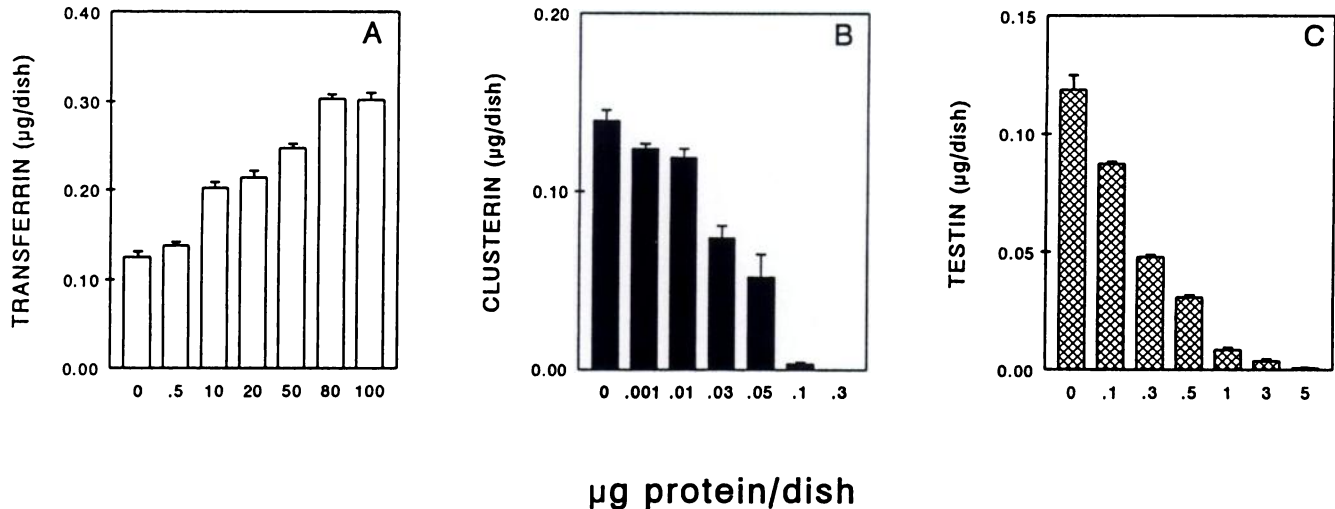


FIG. 2. (A–C) Dose-dependent stimulation of SC transferrin secretion and inhibition of SC clusterin and testins by GC-conditioned media. Values are mean \pm SEM of four replicate determinations from a representative experiment.

SPC-conditioned media because some defined stages of GC such as residual bodies/cytoplasts from elongated spermatids may not produce any proteins and GC produce proteins according to their stages of differentiation (Monesi, 1967; Meistrich et al, 1981; Parvinen, 1982). It is also known that GC at the pachytene stage of meiosis synthesize proteins more actively than other stages (Monesi, 1967).

Modulation of SC Secretory Function by Proteins from Total GC-Conditioned Medium

Bioassays showed that there were biological activities in GC-conditioned medium that stimulated transferrin secretion (Fig. 2A) and inhibited clusterin and testins secretion (Fig. 2B,C). In each instance, the response of SC was dose dependent on the amount of GC medium added. Based on the mass of GC protein required to produce a half maximal response, it appeared that the activity of the clusterin suppressor (0.03 µg germ cell protein/dish) was approximately 10 times that of the testins suppressor (0.2 µg/dish), which in turn was approximately 100 times more active than the transferrin stimulator (20 µg/dish).

Fractionation of Proteins in GC-Conditioned Medium by Anion-Exchange HPLC

Proteins from GC-, SPT-, and SPC-conditioned media were fractionated by anion-exchange HPLC using an analytical Mono Q column. A total of 15, 17, and 23 major protein peaks were resolved from these media, respectively (Fig. 3A–C). Analysis of the fractions under the protein peaks from these fractionations by SDS-PAGE showed a protein pattern similar to one another (Fig. 3D–F). We have labeled some of the major proteins that are present in conditioned media derived from GC, SPT, and

SPC, with letters a through g (Fig. 3D–F) to illustrate their similarity.

Using anion-exchange HPLC, all three of the bioactivities, namely transferrin stimulator, clusterin inhibitor, and testins inhibitor, in GC-conditioned medium as shown in Figure 2A–C were eluted under protein peaks 1–3 shown in Figure 3A (see Fig. 4 for details). Similarly, the transferrin stimulatory and the clusterin inhibitory activities in SPT-conditioned medium also eluted in protein peaks 1–3 (Fig. 3B), and no significant testin inhibitory activity was identified in these fractions (data not shown). Because all of the biological factors eluted at a similar place on the anion-exchange column in the same fractions, it was important to see if they could be separated on other columns. GC-conditioned medium was used in all subsequent studies.

Sequential Fractionation of Biological Factors in GC-Conditioned Medium that Affect SC Secretory Function

Anion-Exchange HPLC—To further characterize the different biological activities, 11 L of GC-conditioned medium containing 55 mg of protein were fractionated by anion-exchange HPLC onto a preparative Mono Q HPLC column (HR 10/16, 16 × 100 mm, i.d.). The chromatogram and the protein patterns of individual fractions on SDS-polyacrylamide gels from the preparative fractionation were virtually identical to that obtained by analytical fractionation shown in Figure 3A. When aliquots from each of these fractions were bioassayed, two distinctive peaks of transferrin stimulatory activity, designated I and II, were observed (Fig. 4A). Single broad peaks of clusterin and testin inhibitory activities were noted (Fig. 4B,C).

Gel Permeation HPLC—The active fractions that af-

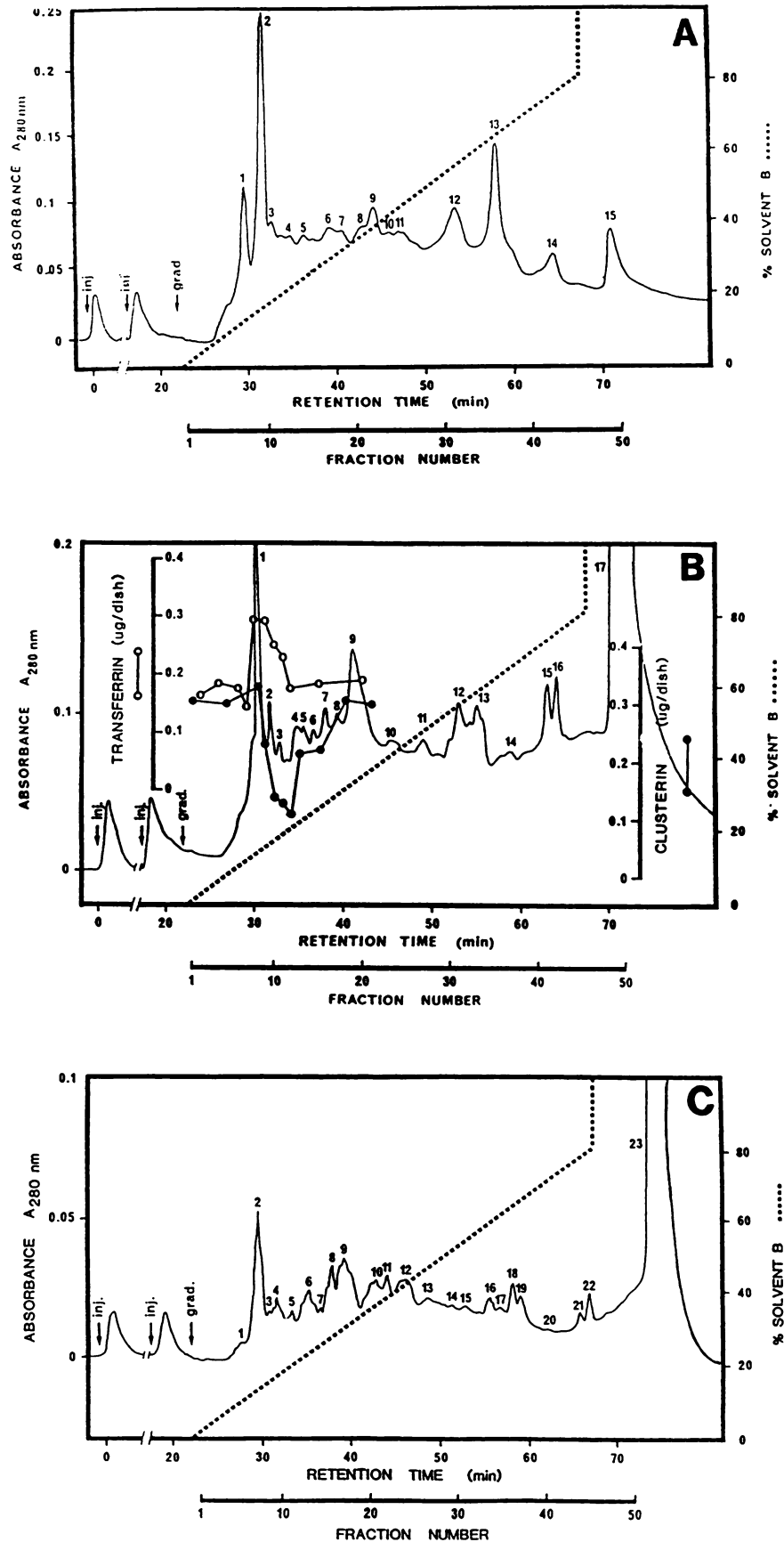
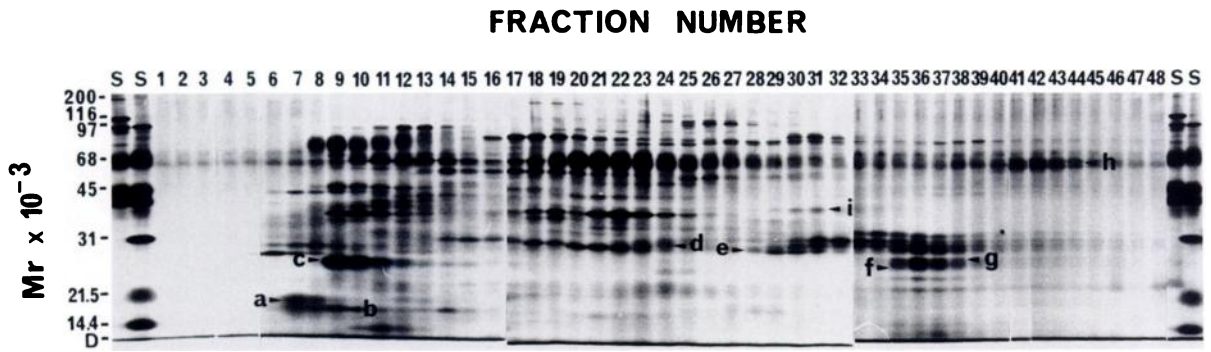
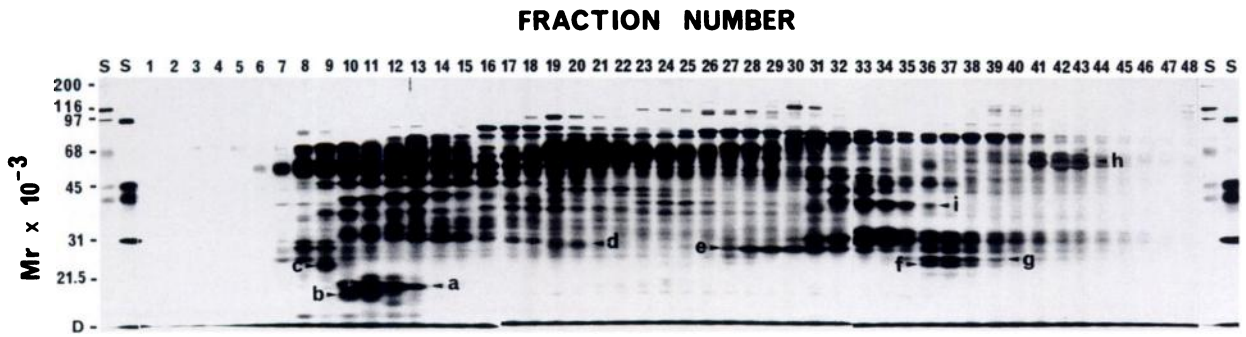


FIG. 3. Fractionation of proteins contained in conditioned media derived from GC, SPT, and SPC by anion-exchange HPLC. **(A)** GC-conditioned media (500 ml). **(B)** SPT-conditioned media (1,000 ml); an aliquot (40 μ l) was bioassayed for its effects on transferrin and clusterin secretion by SC; only fractions with significant bioactivities are plotted. **(C)** SPC-conditioned media (250 ml). **(D–F)** An aliquot (50, 15, and 40 μ l, respectively) from each of the fractions shown in A, B, and C was withdrawn for SDS-PAGE on 10% TSDS-polyacrylamide gels and stained with silver nitrate (lanes 1–48). For comparison purposes, we labeled some of the proteins and designated them a–g. It is noted that these experiments were repeated using two different batches of samples and similar results were obtained in each instance. Lane S, molecular weight markers from BioRad containing 0.2 μ g protein each of myosin Mr 200,000; β -galactosidase, Mr 116,000; phosphorylase b, Mr 97,000; BSA, Mr 68,000; ovalbumin, Mr 45,000; carbonic anhydrase, Mr 31,000; soybean trypsin inhibitor, Mr 21,500; and lysozyme, Mr 14,400. D, dye front.

D



E



F

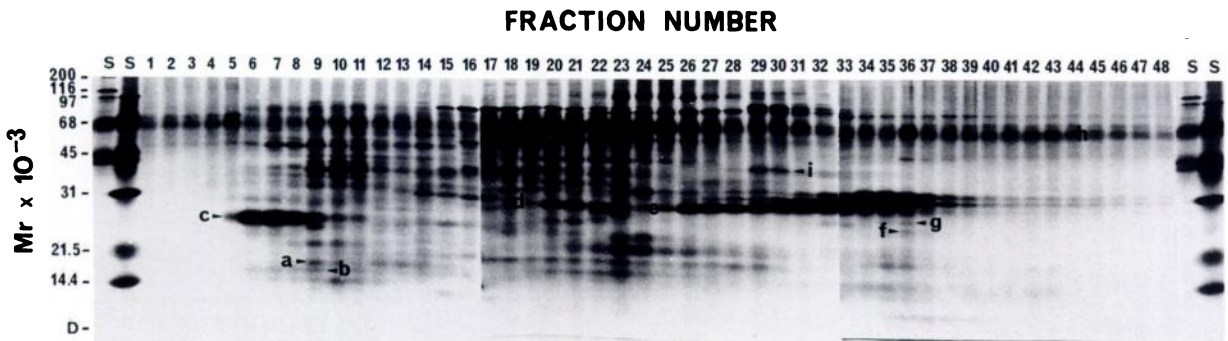


FIG. 3. Continued.

ected both SC transferrin (peak I) and clusterin secretions (fractions 6–12) shown in Figure 4 were pooled, concentrated, and further fractionated by gel permeation HPLC. Nine protein peaks were observed (Fig. 5A). When an aliquot from some of these fractions was visualized by SDS-PAGE and silver staining (Fig. 5B), multiple proteins were noted. Bioassays using aliquots from these fractions showed that the transferrin stimulatory bioactivity (peak I) was associated with a single peak in fractions 15–20 (Fig. 6A). By contrast, there were three separate peaks of clusterin inhibitory activity designated A, B, and C (Fig. 6B). Peak I of transferrin stimulator corresponded to peak B of the clusterin inhibitor.

Reversed-Phase HPLC—The active fractions that affected both SC transferrin (peak II) and testins secretion (fractions 13–27) shown in Figure 4 were pooled and further fractionated by reversed-phase HPLC. A total of 27 protein peaks were seen (Fig. 7A). When an aliquot from some of these fractions was resolved by SDS-PAGE onto 12.5% T SDS-polyacrylamide gels and stained with silver nitrate (Fig. 7B), multiple proteins were noted. When an aliquot from each of these fractions was withdrawn and bioassayed, the transferrin stimulatory activity was detected in three peaks: fractions 50–54 (IIa), fractions 61–65 (IIb), and fractions 67–76 (IIc) (Fig. 8A). Two peaks of testins inhibitory activity were noted and designated 1 and 2 (Fig. 8B) that corresponded to peaks IIb and IIc of transferrin stimulators.

Discussion

Due to the presence of the blood–testis barrier formed by the junctional complexes between adjacent SC, essential elements required for spermatogenesis cannot diffuse to the seminiferous tubular lumen from the systemic circulation; thus, many of the required macromolecules must be either synthesized by SC or other testicular cells *in vivo*. It has been postulated that the transferrin synthesized and secreted by SC plays an important role in the spermatogenic processes by transporting iron to meiotic and post-meiotic GC (Skinner and Griswold, 1980, 1982). It has been proposed that SC bind and internalize diferric transferrin at their basal pole (Huggenvik et al, 1985; Morales and Clermont, 1986) where iron is subsequently transported to the GC in the adluminal compartment of the seminiferous tubules (Morales et al, 1987b). Moreover, adluminal GC such as SPC and SPT possess specific receptors for transferrin (Holmes et al, 1983; Sylvester and Griswold, 1984; Brown, 1985). Several studies indicate that the regulation of SC transferrin is controlled by various factors such as hormones and vitamin A (Huggenvik et al, 1985, 1987; Perez-Infante et al, 1986; Hugly and Griswold, 1987). In addition, the synthesis of transferrin

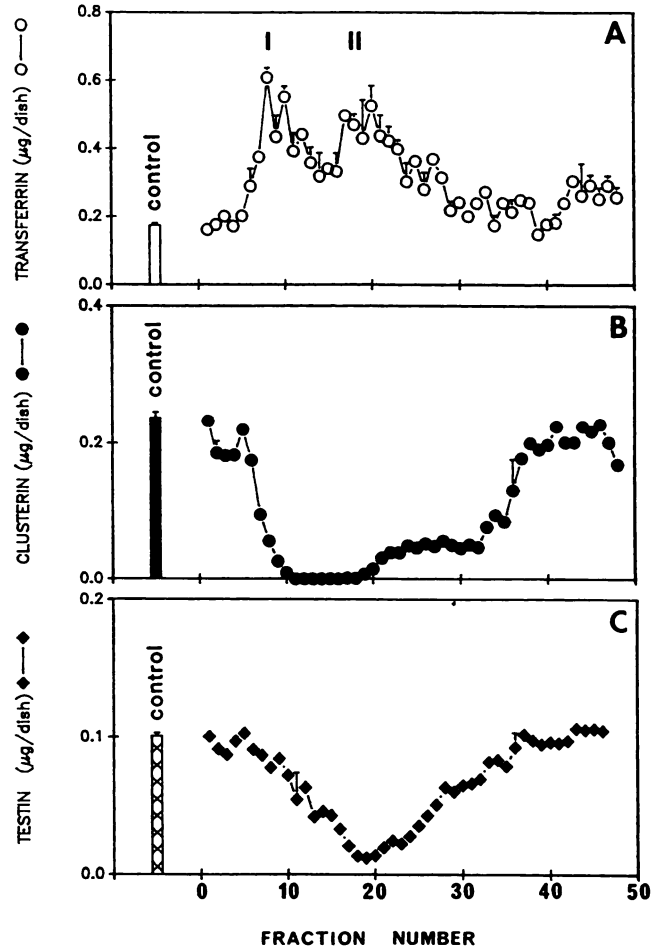


FIG. 4. Bioassay of the GC factor(s) contained in GC-conditioned media that affect SC secretory functions. An aliquot of 20, 2, and 2 μ l from each of the anion-exchange HPLC fractions (Fig. 3A) was bioassayed for its effects on SC transferrin (A), clusterin (B), and testins (C) secretion, respectively. Values are mean \pm SEM of three replicate determinations.

by SC varies with the stages of the seminiferous epithelium (Morales et al, 1987a).

In our studies, it was noted that the transferrin stimulatory activity is detected in the total GC-conditioned medium, which is in agreement with a previous study (Le Magueresse et al, 1988) which demonstrated that GC exert a stimulatory effect on SC transferrin secretion using GC–SC cocultures. This earlier study also demonstrated that SPT- and to a lesser extent SPC-conditioned media are capable of stimulating transferrin secretion via diffusible factor(s). Our results are also in agreement with recent findings showing that SPT and SPC protein(s) stimulate SC transferrin secretion *in vitro* (Onoda and Djakiew, 1990, 1991). Four distinct stimulatory activities, designated I, IIa, IIb, and IIc, were found in GC-conditioned media suggesting the influence of several GC factors on SC transferrin production.

Clusterin is a heterogeneous glycoprotein consisting of

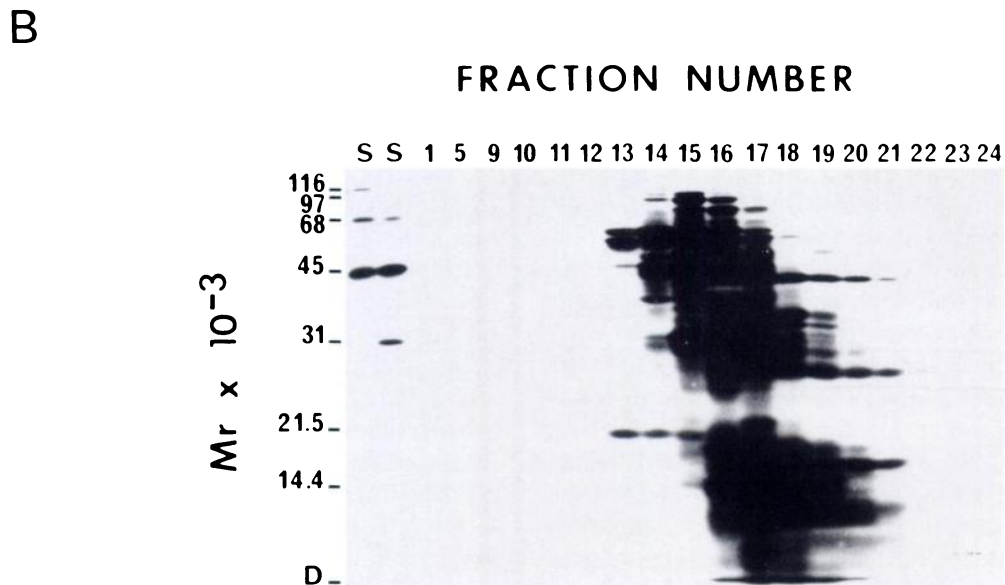
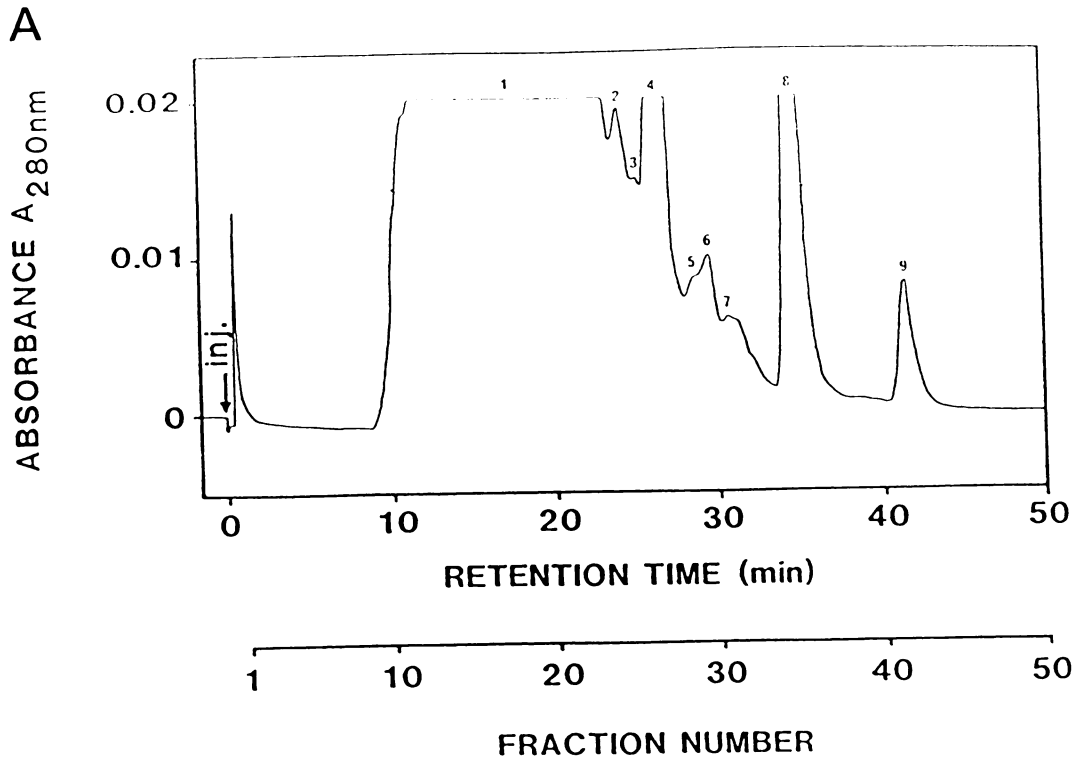


FIG. 5. Fractionation of biological factors contained in GC-conditioned media. **(A)** The biologically active fractions under peak I (shown in Fig. 4A) that modulate SC transferrin and clusterin secretions were fractionated by gel permeation HPLC. **(B)** An aliquot (10 μ l) from each of these fractions was withdrawn for SDS-PAGE and resolved on a 12.5% T SDS-polyacrylamide gel and stained with silver nitrate (lanes 1–24). Lane S, molecular weight markers. D, dye front.

two dissimilar subunits that also designated sulfated glycoprotein-2 (SGP-2; Collard and Griswold, 1987; Cheng et al, 1988). Studies on the tissue distribution of clusterin indicate that this protein is present in almost all organs examined (Cheng et al, 1990; Mathur et al, 1990). In the testis, clusterin is a major protein secreted by SC (Kissinger et al, 1982). However, its function in the seminiferous epithelium has not yet been established. Due to its sequence homology with apolipoprotein (Collard and Griswold, 1987), it was postulated that this protein may be involved in GC membrane biogenesis. The identification of clusterin over the acrosome and tail of elongating spermatids and mature spermatozoa (Sylvester et al, 1984; Kierszenbaum et al, 1988) suggests that this protein may also be associated with sperm maturation. This protein can also induce aggregation of spermatozoa and other cells (Fritz et al, 1989) probably because of its high carbohydrate content.

Studies using an *in vitro* bicameral culture system have showed that SC secrete clusterin predominantly into the apical compartment and that its secretion is modulated by GC (Djakiew and Dym, 1988; Onoda and Djakiew, 1991). The conclusion that GC regulate clusterin secretion was supported by transillumination and microdissection techniques showing cyclic variation in clusterin release from tubular segments in different stages of spermatogenesis (Shabanowitz et al, 1986). Recent studies from our laboratory have shown that total GC-conditioned media inhibited the apical secretion of this protein dose dependently (Grima et al, 1992). In the present study, three separate inhibitory activities were found in GC-conditioned media and were designated A, B, and C. Peak B of the clusterin inhibiting activity corresponds to peak I of the transferrin stimulating activity.

Testins I and II are two monomeric proteins originally designated CMB-22 and CMB-23. These proteins possess similar physicochemical and immunological properties (Cheng and Bardin, 1987). These two proteins are virtually identical except that CMB-23 has three extra NH₂-terminus amino acids of A-A-P- (Cheng et al, 1989; Cheng et al, 1993). Previous studies from this laboratory have demonstrated that SC synthesize and secrete these proteins *in vitro* (Cheng et al, 1989; Grima et al, 1992) and that their secretion is stimulated by testosterone (Cheng and Bardin, 1987). The intact tubule secretes only small amounts of testins, but there is a rise in testicular testin levels following the disappearance of GC in busulfan or X-ray treated rats (Cheng et al, 1989; Jégou et al, 1992; Cheng et al, 1993). These observations revealed an inverse correlation between the presence of GC, and particularly of late spermatids, and testicular levels of these proteins. The present study indicates that there are two separate peaks of inhibitory activity (designated 1 and 2) in GC-

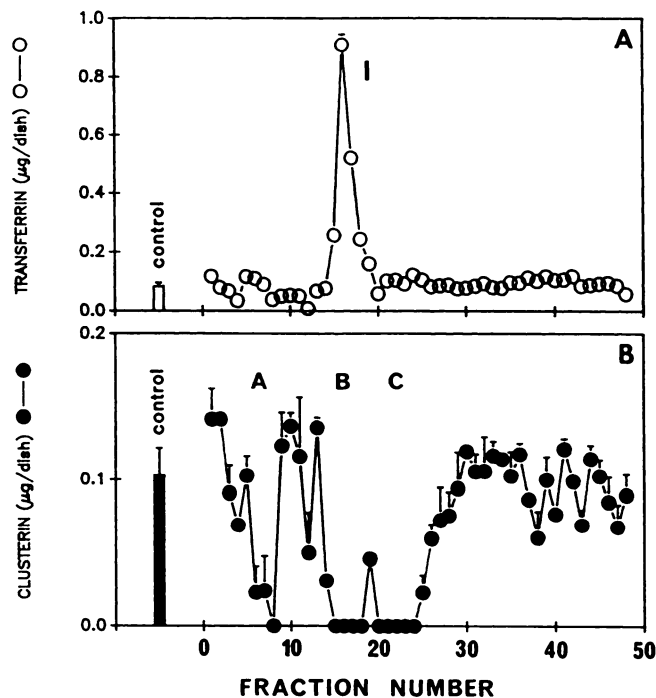


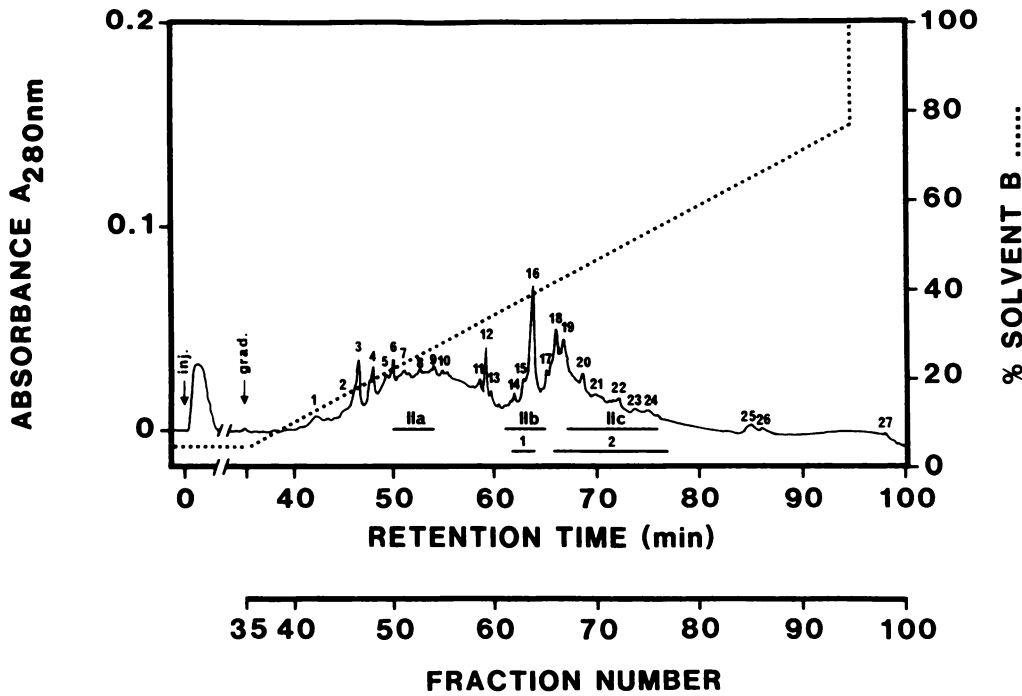
FIG. 6. Bioassay of the GC factors fractionated by gel permeation HPLC for their effects on SC secretory functions. An aliquot (10 μ l) from each of the fractions shown in Figure 5A was bioassayed using SC-enriched cultures for its effects on SC transferrin (A) and clusterin (B) secretion. Values are mean \pm SEM of three replicate determinations.

conditioned media. These peaks corresponded with peaks IIb and IIc of transferrin stimulating activity, respectively.

Our present data are not in complete agreement with other results showing a dose-dependent stimulation of SC clusterin secretion using conditioned media derived from SPT (Onoda and Djakiew, 1990) and SPC (Onoda and Djakiew, 1991). These authors have also described a dose-dependent stimulation of testis secretion using conditioned media derived from SPT (Onoda and Djakiew, 1990). The observed discrepancy may be due to the differences of the culture conditions used for these studies and also in the amounts of GC proteins used. These earlier studies have used GC cultured for 3 days at 34°C that had a cell viability of 4–7% at the end of the incubation period. Thus, dead cells could release cytosolic components into the media following cell lysis. In our studies, total GC and defined stages of GC were cultured for a relatively short period of time (i.e., 20 hours), and the final preparations have a cell viability of about 90%. It has been shown that there is a positive correlation between the biological activity of GC-conditioned medium and the viability of the cells (Le Magueresse and Jégou, 1988a).

Moreover, the inhibition of clusterin and testis secretion by GC-conditioned medium is not a nonspecific effect due to subsequent SC death in the cultures since when these same spent media were quantified for transferrin,

A



B

FRACTION NUMBER

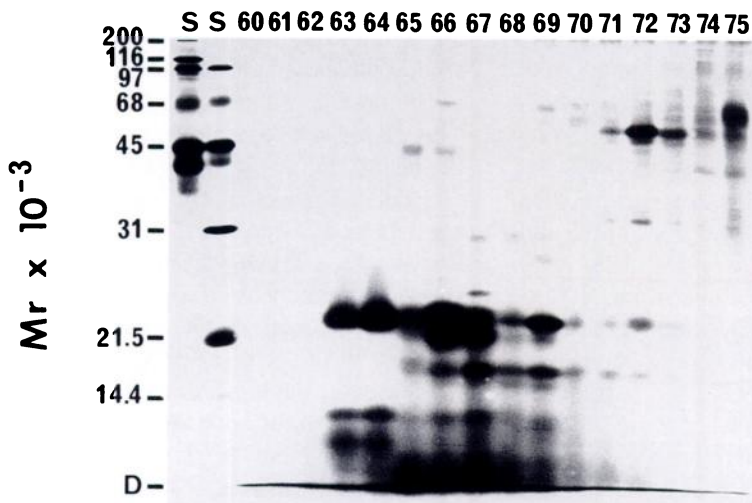


FIG. 7. Fractionation of the biological factors contained in GC-conditioned media that modulate SC transferrin (peak II, shown in Fig. 4A) and testis secretion by reversed-phase HPLC. **(A)** Sample was fractionated by reversed-phase HPLC using the procedure outlined in *Materials and Methods*. **(B)** An aliquot (15 μ l) from some of these fractions was withdrawn for SDS-PAGE on a 14% T SDS-polyacrylamide gel and stained with silver nitrate (lanes 60–75). Lane S, molecular weight markers. D, dye front. IIa, IIb, IIc represent the three distinctive peaks of biological activity for the transferrin stimulator; 1, 2 represent the two distinctive peaks of biological activity for the testis inhibitor.

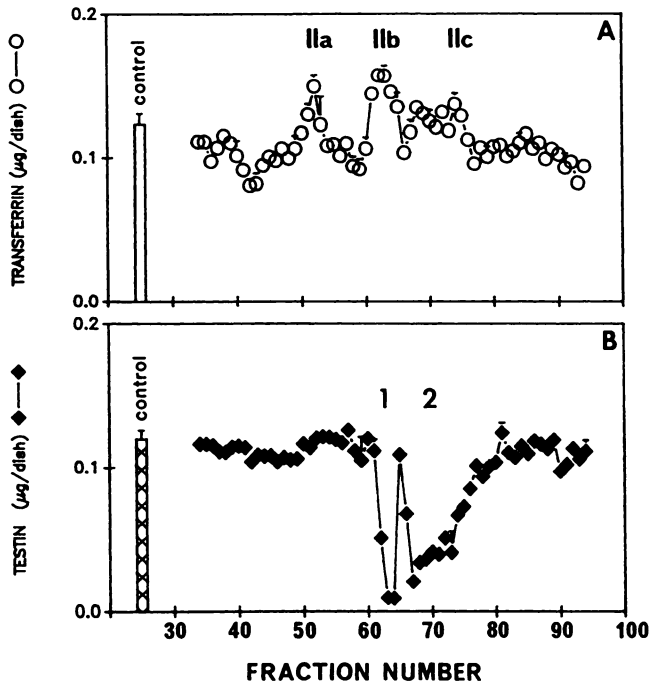


FIG. 8. Bioassay of the GC factors fractionated by reversed-phase HPLC. An aliquot (20 μ l) from each of the fractions was bioassayed using SC-enriched cultures for its effects on transferrin (A) and testin (B) secretion. Values are mean \pm SEM of three replicate determinations.

they showed an increase in transferrin secretion. Also, the reported inhibitory activity could not be attributed to the additives contained in the GC-conditioned medium because media prepared in the absence of GC had no apparent effect on SC transferrin, clusterin, and testins secretion.

This study suggests that three peaks are associated with at least two common bioactivities: I and B; I Ib and 1; and I Ic and 2. However, additional studies have to be performed in order to verify this hypothesis. Furthermore, A, C, and I Ia are associated with only one of the assayed activities. Therefore, six active fractions have been identified.

In summary, the results of the present study support the hypothesis that GC play an important role in regulating SC functions. We have also identified multiple biological factors in GC-conditioned medium that modulate the secretion of transferrin, clusterin, and testins by SC. This study described a strategy for the identification of such biological factors. The purification of putative GC factors is crucial to study the physiological interrelationships between GC and SC in the seminiferous epithelium.

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