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Sertoli Cells Synthesize and Secrete a Ceruloplasmin-Like Protein

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ABSTRACT

Sertoli cells synthesize and secrete a ceruloplasmin-like protein (testicular ceruloplasmin) that is immunologically similar to serum ceruloplasmin. Rat serum ceruloplasmin was purified and an antiserum was produced to the purified protein which specifically immunoprecipitated a 130,000 dalton protein from rat serum. This ceruloplasmin antiserum was found to also immunoprecipitate a 130,000 dalton protein synthesized and secreted by Sertoli cells. The presence of a protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was required during the immunoprecipitation procedure to prevent the proteolytic degradation of testicular ceruloplasmin. Immunoprecipitation of proteins secreted by Sertoli cells with an antiserum to rat serum proteins was found to precipitate two proteins, testicular ceruloplasmin and testicular transferrin.

INTRODUCTION

Within the seminiferous tubules is a specialized serum-free environment whose composition is determined primarily by the Sertoli cells (Waites, 1977). Many of the macromolecules required for somatic cell maintenance and development which are present in serum are not available to the developing germinal cells within the tubules. Serum-like proteins which are required for germinal cell viability are probably synthesized and secreted by Sertoli cells. The ability to isolate and maintain Sertoli cells in serum-free cell culture allows for the examination of the proteins which Sertoli cells secrete. We have demonstrated that cultured Sertoli cells secrete a transferrin-like protein (Skinner and Griswold, 1980). The secretion of testicular transferrin was shown to be regulated by follicle-stimulating hormone (FSH), insulin, testosterone and Vitamin A (Skinner and Griswold, 1982).

Immunoprecipitation of proteins secreted by cultured Sertoli cells with antiserum to rat serum proteins was found to precipitate a protease-sensitive 130,000 dalton protein in addition to testicular transferrin (Skinner and Griswold, 1980). Ceruloplasmin is a protease-sensitive serum protein of 130,000 daltons which is the major copper transport protein in serum (Frieden and Hsien, 1976). Ceruloplamsin, which acts as a serum oxidase, has also been shown to aid in the formation of FE(III) transferrin (Mareschal et al., 1980). It is thus possible that ceruloplasmin is a protein which is required for germinal cell viability.

In the present study, serum ceruloplasmin was purified and an antiserum was then produced. This ceruloplasmin antiserum was then utilized to determine that Sertoli cells secrete a ceruloplasmin-like protein.

MATERIALS AND METHODS

Chemicals

Medium for cell culture (Ham's F-12) was made from a powdered formulation supplied by Gibco. Rabbit anti-rat transferrin antiserum was obtained from Cappel Labs. [^{35}S]Methionine (approx. 1000 Ci per mmole) was obtained from New England Nuclear Corp. Formalin-fixed *Staphylococcus aureus* (Pansorbin) was obtained from Calbiochem-Behring Corp. All other chemicals were obtained from Sigma Chemical Co.

Cell Culture

Sertoli cells from 20-day-old rats were prepared and cultured as previously described (Kissinger et al., 1982; Dorrington and Fritz, 1975). Cells were cultured in 24-well dishes (Linbro Scientific Co.). Approximately 5×10^5 cells were plated per well and the cells were maintained in the absence of calf serum and presence of dibutyryl cyclic AMP (0.1 mM) and testosterone (0.7 mM). The medium (1 ml) from each well was collected and replenished every 2 days for the duration of culture. To obtain radioactive-secreted protein, 10 μCi of [^{35}S]methionine was added per milliliter of medium lacking methionine on Day 5 of culture and the medium was collected on Day 6 of culture. This procedure was chosen because we have

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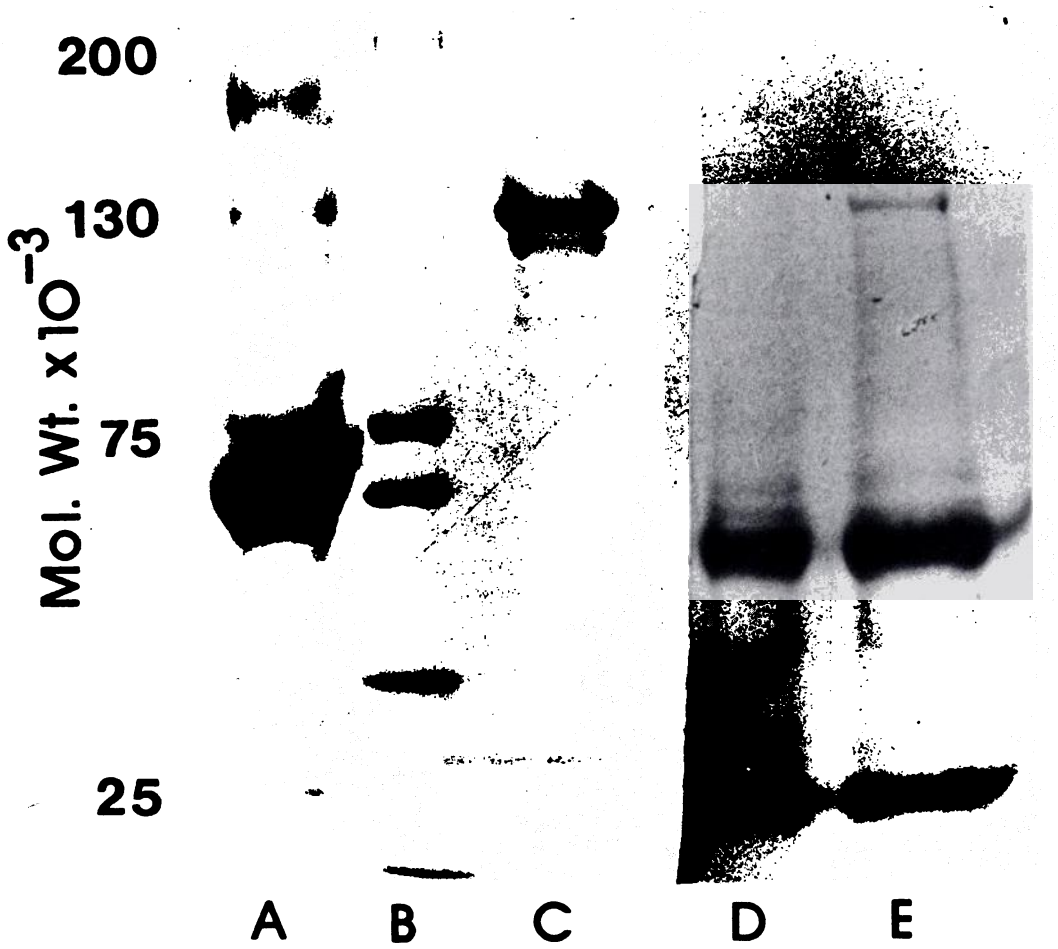

FIG. 1. Analysis of the purified rat serum ceruloplasmin and the ceruloplasmin antiserum on an SDS-polyacrylamide gradient slab gel stained with Coomassie blue. The lanes are: *A*) rat serum; *B*) molecular weight standards of ferritin, transferrin, albumin, and lactic dehydrogenase; *C*) purified rat serum ceruloplasmin; *D*) control double antibody immunoprecipitation using nonimmune rabbit seurm; and *E*) immunoprecipitation of rat serum with anti-ceruloplasmin antibody.

previously shown that it will lead to maximum incorporation of isotope into testicular transferrin (Skinner and Griswold, 1982). Peritubular fibroblasts were prepared and cultured as previously described (Kissinger et al., 1982). Radiolabeled proteins secreted by peritubular cells were obtained using the same procedure as described above.

Ceruloplasmin Isolation and Antiserum Production

Rat serum ceruloplasmin was isolated as previously described by Noyer (1980). Rat serum was collected and frozen at −80°C, and then thawed at 4°C. Prior to freezing, PMSF (phenylmethylsulfonyl fluoride, 57 μM) was added. The thawed serum was centrifuged at 10,000 × *g* for 30 min. The supernatant was made 20% in polyethylene glycol 4000 and stirred at 4°C for 2 h and centrifuged. The supernatant was dialyzed overnight in 0.05 M NaCl, 0.05 mM $CuSO_4$, 0.015 M phosphate pH 6.9. The sample was applied to a DEAE-Sephadex ion exchange column and ceruloplasmin was eluted with a 0.05 to 0.3 M NaCl gradient. Ceruloplasmin elution was monitored spectrophotometrically at 610 nm. The ceruloplasmin peak was pooled and dialyzed against the previous buffer. The sample was applied to a hydroxyapatite column and eluted with a 0.07 M to 0.5 M phosphate gradient (pH 6.9). Elution of ceruloplasmin was monitored spectrophotometrically at 610 nm and the composition of the peak was analyzed with sodium dodecyl sulfate (SDS) gel electrophoresis. Antiserum to this purified rat serum ceruloplasmin was produced in a rabbit. Four injections, each 2 weeks apart, of 0.5 mg of protein were made. The first two injections were in an emulsion of Freund's complete adjuvant and the last two in incomplete adjuvant.

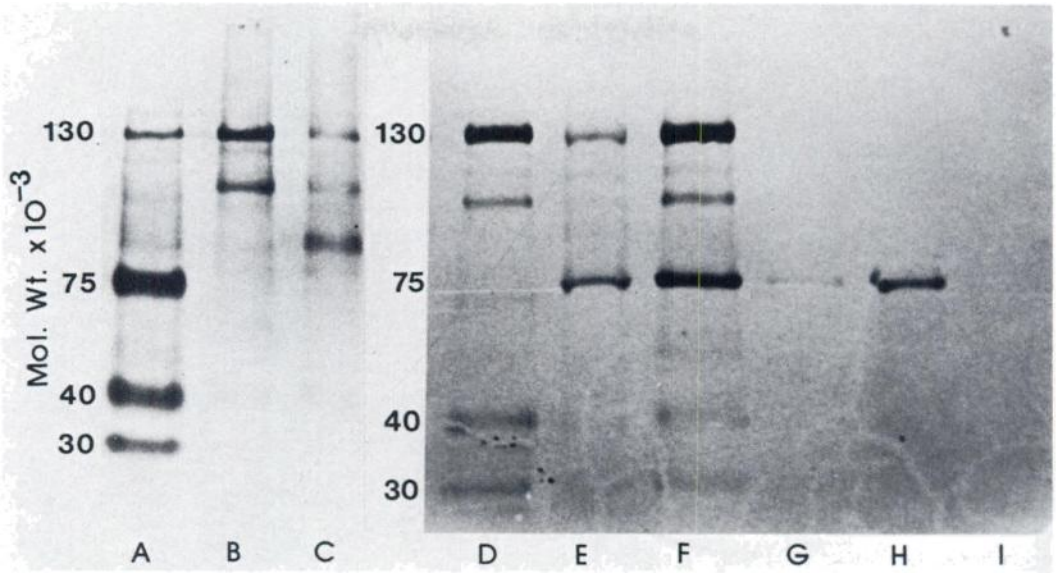

FIG. 2. Immunoprecipitates of [^{35}S] methionine-labeled Sertoli cell-secreted proteins. Fluorographs of SDS-polyacrylamide gradient slab gels are shown. Lanes are: *A*) Sertoli cell-secreted proteins; *B* and *D*) immunoprecipitate of Sertoli cell-secreted proteins with anti-ceruloplasmin and PMSF; *C*) same as *B* but with no PMSF; *E* and *F*) immunoprecipitate of Sertoli cell-secreted proteins with anti-rat serum antibodies; *G* and *H*) immunoprecipitate of Sertoli cell secreted proteins with anti-transferrin antibodies; and *I*) control immunoprecipitate using nonimmune rabbit serum.

Electrophoresis

SDS polyacrylamide gel electrophoresis was done according to the Laemmli procedure (1970). Gradient slab gels of 5 to 15% acrylamide were used. For fluorography, slab gels were incubated for 1.5 h in a 20% (w/v) 2,5-diphenyloxazole (PPO)-acetic acid solution and then for 30 min in water (Kissinger et al., 1982). The gels were dried and exposed to preflashed X-Omat Kodak x-ray film.

Immunoprecipitations

Precipitation of immune complexes was accomplished with the use of *Staphylococcus aureus* (Pansorbin) fixed with formalin (10% w/v). To 100 μl of sample, 200 μl of buffer [50 mM Tris, 0.15 M NaCl, 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, pH 7.5] and 5 μl of nonimmune rabbit serum were added and then the mixture was incubated at 4°C for 4 h. Pansorbin (100 μl) was added, the sample incubated for 4 h at 4°C and centrifuged. The supernatant was collected, 25 μl of antiserum was added and the mixture was incubated overnight at 4°C. Pansorbin (100 μl) was added again and the sample was incubated for 4 h at 4°C. The samples were then centrifuged and the pellet was washed five times with 200 μl of buffer. The pellet was heated for 30 min in SDS sample buffer on a steam bath with occasional mixing prior to centrifugation and application of the sample to the SDS polyacrylamide slab gel.

Because Pansorbin can add to the number of Coomassie blue-stained bands on a gel, nonradioactive serum ceruloplasmin was immunoprecipitated by a different method. The procedure for this immunoprecipitation consisted of two 24-h incubations at 4°C, first with the anti-ceruloplasmin and second with a goat anti-rabbit serum. The sample was then centrifuged and washed.

RESULTS

Ceruloplasmin was isolated from rat serum by an established procedure and analyzed with SDS polyacrylamide gel electrophoresis (Figs. 1A and C). The ceruloplasmin preparation was found by gel electrophoresis to contain primarily a protein band with an apparent molecular weight of 130,000. In addition, several lower molecular weight proteins were found in the preparation in low concentration. These proteins probably result from proteolytic degradation of ceruloplasmin. This rat serum ceruloplasmin preparation was then used to produce an antiserum in a rabbit.

SDS polyacrylamide gel electrophoresis was used to analyze immunoprecipitations done with anti-rat transferrin, anti-rat serum proteins, and anti-rat ceruloplasmin antisera. The antiserum to ceruloplasmin was used to immunoprecipitate a protein in rat serum (Fig. 1E). A control was run in which nonimmune rabbit

serum was used to replace the ceruloplasmin antiserum and only the 50,000 and 25,000 dalton fragments of the immunoglobulins were precipitated (Fig. 1D). As shown, the ceruloplasmin antiserum specifically precipitated a 130,000 dalton protein from rat serum.

Immunoprecipitates of radioactive proteins secreted by Sertoli cells are shown in Fig. 2. The antiserum to rat serum ceruloplasmin immunoprecipitated a 130,000 dalton Sertoli cell-secreted protein and one other 100,000 dalton peptide. If PMSF was omitted during the immunoprecipitation procedure, an additional proteolytic fragment of ceruloplasmin was detected (Fig. 2C). Antiserum to serum transferrin immunoprecipitated the 75,000 dalton protein previously characterized as testicular transferrin (Skinner and Griswold, 1980). Antiserum to rat serum proteins was found to precipitate both the testicular transferrin and the 130,000 dalton polypeptide.

Peritubular cells are the most common contaminate of Sertoli cell culture. Radioactive proteins secreted by peritubular cells were obtained from subcultured peritubular cells. These cultures generally contain more than 90% peritubular cells and only a small number of Sertoli cells. Immunoprecipitation of proteins secreted by peritubular cell cultures with an antiserum to rat serum proteins is shown in Fig. 3. Several proteins of high molecular weight are apparently secreted by these cells and are immunoprecipitated with antiserum to rat serum proteins. Only trace amounts of ceruloplasmin or transferrin can be immunoprecipitated from these cultures. This low amount of protein can probably be accounted for by the presence of a small number of Sertoli cells.

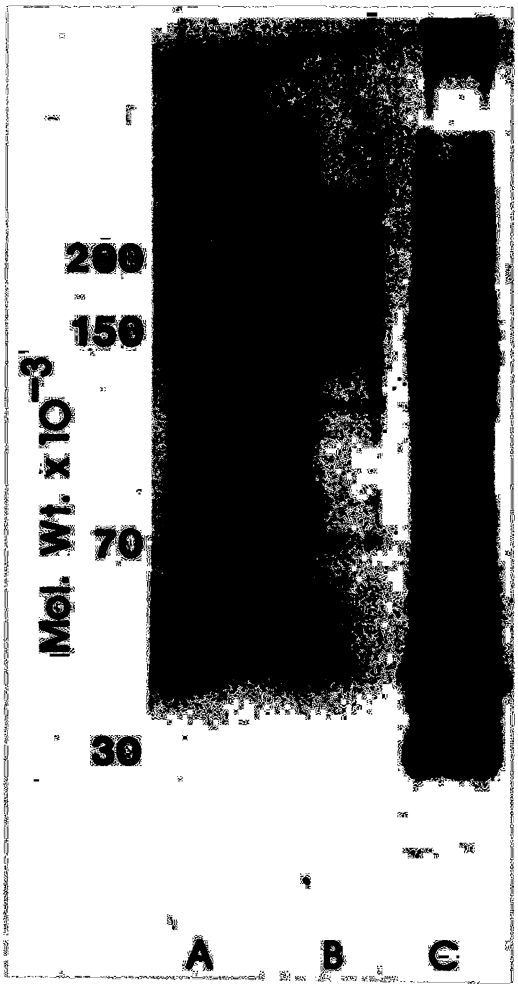

FIG. 3. Peritubular cell-secreted serum-like proteins. Fluorograph of [^{35}S]methionine-labeled peritubular cell-secreted proteins analyzed on an SDS-polyacrylamide gradient slab gel. Lanes are: *A* and *B*) immunoprecipitation of peritubular cell-secreted proteins with anti-rat serum antibodies; and *C*) total peritubular cell-secreted proteins.

DISCUSSION

The antiserum used in these studies was specific to ceruloplasmin. The serum ceruloplasmin used as an antigen was purified by a published procedure in which the copper binding protein was monitored by absorption at 610 nm (Noyer et al., 1980). This procedure yielded a product which revealed only a single band at 130,000 daltons after gel electrophoresis. The antiserum to this protein was used to immunoprecipitate a single protein of 130,000 daltons from freshly prepared rat serum.

Immunoprecipitation of proteins secreted by Sertoli cells with the ceruloplasmin antiserum resulted in the precipitation of the 130,000 dalton secreted protein. Also precipitated was a 100,000 dalton peptide. It has been shown that ceruloplasmin is protease sensitive and that a 100,000 dalton peptide is a common proteolytic fragment (Noyer et al., 1980). For this reason we suggest that the 100,000 dalton fragment precipitated is a proteolytic fragment of the 130,000 dalton protein. This peptide may be generated during cell culture due to proteases in the medium or during immunoprecipitation due to proteases in the antisera. Since a protease inhibitor (PMSF) eliminated several polypep-

tides which were precipitated with the ceruloplasmin antiserum, it is possible that those polypeptides were generated by proteases during the immunoprecipitation procedure.

Immunoprecipitation of proteins secreted by Sertoli cells with an antiserum to rat serum proteins precipitated both transferrin and ceruloplasmin. In the presence of PMSF a 75,000, a 100,000 and a 130,000 dalton protein were precipitated. These results correlate with those previously reported in which a 75,000 and 130,000 dalton proteins were precipitated (Skinner and Griswold, 1980). Clearly, testicular ceruloplasmin and testicular transferrin are the most readily detected serum-like proteins secreted by Sertoli cells. These cells may secrete other serum-like proteins but these proteins would necessarily be secreted at very low levels or would be similar to trace serum components. Recently, Wright et al. (1981) have shown by an immunoprecipitation procedure which was similar to the one used in this study, that as many as 7 to 9 serum-like proteins were precipitated from Sertoli cell-secreted proteins with an antiserum to rat serum proteins. However, the authors did not use any protease inhibitors before or during the immunoprecipitation procedure. Also, it is clear that peritubular cells secrete some high molecular weight proteins which are precipitated with antiserum to rat serum proteins (Fig. 3). Since the most common cell contaminate in Sertoli cell cultures are peritubular cells, the presence of significant numbers of peritubular cells in those cultures could increase the number of serum-like proteins detected.

Serum ceruloplasmin can function in copper transport and act as a ferroxidase. Since the developing germinal cells are in a serum-free environment the function of a testicular ceruloplasmin may include both of these functions.

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