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Purification of a Paracrine Factor, P-Mod-S, Produced by Testicular Peritubular Cells That Modulates Sertoli Cell Function*

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A testicular paracrine factor, P-Mod-S, was purified from conditioned medium obtained from serum-free cultures of peritubular cells. Stimulation of testicular transferrin production by cultured Sertoli cells was utilized as a bio-assay for P-Mod-S. A bioactive protein with an apparent molecular weight of 50,000 under physiological conditions was isolated by high pressure size exclusion chromatography. P-Mod-S was found to have an affinity for heparin and bound to a heparin affinity column. Two forms of P-Mod-S were purified with reverse-phase chromatography. The less hydrophobic form was referred to as P-Mod-S (A) and is a 56,000 molecular weight protein. The more hydrophobic form was referred to as P-Mod-S (B) and is a 59,000 molecular weight protein. Purification of P-Mod-S (A) and P-Mod-S (B) from peritubular cell-radiolabeled secreted proteins revealed that both proteins contain radioactivity. This result demonstrates active synthesis and secretion of P-Mod-S by peritubular cells. Although the amino acid composition of the two proteins indicates distinct differences in the content of several amino acids, the relationship of P-Mod-S (A) and P-Mod-S (B) is unknown at present. A greater than 1000-fold increase in the specific activity of P-Mod-S was achieved with the purification procedure utilized. P-Mod-S can account for essentially all the bioactivity present in crude peritubular cell-secreted protein preparations. The effects of the two forms of P-Mod-S on both transferrin and androgen-binding protein production by Sertoli cells was examined. Purified forms of P-Mod-S were found to have a greater effect on Sertoli cell function than any individual regulatory agent previously known to influence the cell, including follicle-stimulating hormone. The significance of peritubular cell-Sertoli cell interactions mediated via P-Mod-S to spermatogenesis and testicular function is discussed, as well as insight provided into general mesenchymal-epithelial cell interactions.

A number of different somatic cell types in the testis have a role in the maintenance and control of spermatogenesis. Sertoli cells form the seminiferous tubules, help create the blood-testis barrier, and provide the cytoarchitectural support for the developing germinal cells. Peritubular (myoid) cells are mesenchymal cells which surround the seminiferous tubule and produce a basement membrane in cooperation with Sertoli cells. Leydig cells are a functional cell in the interstitum responsible for the production of androgens. Interactions between these different cell types are critical for the development and maintenance of testicular function and spermatogenesis.

The transport of essential components to developing germinal cells is an important functional parameter of Sertoli cells. An example of this nutritional type interaction between Sertoli cells and germ cells is the production of energy metabolites by Sertoli cells which can subsequently be utilized by germ cells (1, 2). Other examples are the production of testicular transferrin (3) and ceruloplasmin (4) by Sertoli cells which transport iron and copper to germ cells, respectively (3-6). The production of these proteins, particularly transferrin, provide convenient functional markers for the cell to assess the hormonal regulation of Sertoli cell function (7). Regulatory agents which modulate these Sertoli cell functions will ultimately influence the maintenance and control of germ cell development.

Important environmental and structural interactions occur between Sertoli cells and peritubular cells (8), and a recent report also indicates the presence of a regulatory interaction between these cells. Peritubular cells have been shown to produce a paracrine factor termed P-Mod-S that modulates Sertoli cell function (9). A crude preparation of P-Mod-S can stimulate Sertoli cell function to a greater extent than any agent previously known to regulate Sertoli cell function (9, 10). In addition, androgens appear to stimulate the production of P-Mod-S by peritubular cells (9, 11). Therefore, it is postulated that P-Mod-S may play an integral role in the maintenance and control of Sertoli cell function and provide a mode of androgen action in the seminiferous tubule. In support of this hypothesis co-cultures of Sertoli cells and peritubular cells enhance Sertoli cell function (12, 13) as well as augment the actions of androgens on Sertoli cell function (11). A cellular interaction in the testis is proposed in which Leydig cells in the interstitium produce androgens which can act on peritubular cells to increase or maintain the production of P-Mod-S that then can act on Sertoli cells to modulate functions associated with the maintenance and control of germinal cell development.

Peritubular cell-Sertoli cell interactions provide an example of mesenchymal cell-epithelial cell interactions. Previous studies have proposed the production of inducer substances by mesenchymal cells which alter the development and differentiation of the adjacent epithelial cells (for review, see Ref. 14). This mesenchymal-epithelial cell interaction is speculated to be important during development as well as for the maintenance of normal function in the adult tissue. P-Mod-

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S production by peritublar cells provides an example of a possible mesenchymal inducer substance whose production is under hormonal control. Further analysis of the interaction between peritubular cells and Sertoli cells mediated by P-Mod-S will provide a better understanding of mesenchymal-epithelial cell interactions.

Because of the potential importance of P-Mod-S in the maintenance of spermatogenesis and testicular function, the current study was designed to purify P-Mod-S from peritubular cell-conditioned medium. Two forms of P-Mod-S were isolated which retained the ability to regulate Sertoli cell function.

MATERIALS AND METHODS

Cell Preparation and Culture—Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion (15) with a modified procedure previously described (16). Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml) (Gibco) to remove Leydig cells, followed by a collagenase digestion (1 mg/ml Type I, Sigma) and then a hyaluronidase digestion (1 mg/ml, Sigma). Sertoli cells were then plated in 24-well (1 ml/well) Linbro plates at approximately 5×10^5 cells/well. Cells were maintained at 32 °C in a 5% CO_2 atmosphere in Ham's F-12 medium (Gibco). Sertoli cultures were treated as described under "Results" at the time of plating and retreated after 48 h of culture when the medium was replenished. Unless otherwise stated, a 72-h medium collection on day 5 of culture was obtained for analysis and the cells harvested for a DNA assay. Sertoli cell cultures were treated as outlined under the "Results" section with test substances or with FSH[1] (100 ng/ml), insulin (5 μg/ml), retinol (0.35 μM), and testosterone (1 μM).

Peritubular cells were obtained from the collagenase digestion supernatant after tubule segments had gravity sedimented as previously described (9). Peritubular cells were plated in medium containing 10% calf serum and grown to confluence. Cells were then subcultured and plated at 25% confluence. After 3–4 days of culture, subcultured cells were confluent and washed for 24 h with serum-free medium. The cells were then cultured for up to 2 weeks in serum-free medium with 48-h medium collections.

Freshly collected peritubular cell serum-free conditioned medium was made 25 μM phenylmethylsulfonyl fluoride and 0.1 mM benzamidine and then centrifuged at 1,000 × *g* for 15 min at 4 °C to remove cell debris. When required, medium was frozen and stored at −20 °C. Conditioned medium was concentrated 100-fold by ultrafiltration with an Amicon system using a membrane with a 10,000 molecular weight exclusion limit.

When required peritubular cell cultures were maintained for 48 h starting on day 5 of serum-free culture in glycine and methionine-free media containing 5 μCi/ml [^{35}S]methionine and 5 μCi/ml [^{3}H] glycine. The radiolabeled media was collected, centrifuged, and used for analysis.

Size Exclusion High Pressure Liquid Chromatography (HPLC)—Size exclusion chromatography of concentrated medium from peritubular cells was accomplished with an HPLC apparatus (Beckman). The column utilized was a series of TSK (Toyo-Soda) 7.5 mm × 30 cm columns including one SW 4000, two SW 3000, and one SW 2000. The column was equilibrated and eluted at 0.5 ml/min with 100 mM Na_2SO_4, 50 mM phosphate, 1 mM triethylamine, 0.5% (v/v) ethylene glycol, pH 7.0. Fractions were collected (2 min) and stored at −20 °C before being utilized. Standardization of the column for molecular weights was done with thyroglobulin (660 kDa), bovine serum albumin (68 kDa), ovalbumin (42 kDa), and chymotrypsinogen (25 kDa). With a 0.5 ml/min flow rate this size exclusion column has a K_{av} of 0.0 at a 40-min retention time and a K_{av} of 1.0 at a 120-min retention time.

Reverse-phase High Pressure Liquid Chromatography—Reverse-phase chromatography was performed on an analytical Vydac-C4 column. Unless otherwise stated, the serum was equilibrated in 0.1% (v/v) trifluoroacetic acid, 0.5% (v/v) ethylene glycol, 1 mM triethylamine, pH 2.0. Acidified samples were loaded and eluted at 0.5 ml/min with a linear gradient from 25 to 60% acetonitrile. Fractions were collected (2 min) and dried in a speed-vac apparatus (Savant). Dried samples were then reconstituted and when necessary stored at −20 °C.

Affinity Chromatography—Heparin-Sepharose affinity chromatography was performed on a 1 × 15 cm column equilibrated in 50 mM Tris, pH 7.5. Samples were applied and the column washed with the equilibration buffer. Protein was eluted from the column with 1 M sodium chloride, and 1-ml fractions were collected. Protein elution was monitored at 280 nm absorbance.

Electrophoresis, Silver Staining, and Fluorography—Proteins were electrophoretically analyzed on sodium dodecyl sulfate 5–15% polyacrylamide gradient slab gels under reducing conditions with the Laemmli (17) buffer system. When required the gels were fluorographed with diphenyloxazole in acetic acid as previously described (18).

A silver stain procedure was utilized to detect protein in the gel. Gels were first incubated in 10% (vol/vol) acetic acid, 45% (vol/vol) methanol, 45% water for 18 h then incubated in four successive 30-min washes of water, followed by an incubation in 1% (vol/vol) glutaraldehyde for 2 h with agitation. The gel was then incubated in four successive 30-min washes of water and then incubated for 30 min in the silver stain solution with agitation. The staining solution was made by adding 4 ml of a 1.2 M silver nitrate solution slowly while mixing to 22.5 ml of a 90 mM sodium hydroxide and 0.45 M ammonium hydroxide solution, followed by a dilution to 100 ml with water. After incubation in the silver stain solution, the gel is incubated in three successive 10-min washes of water and transferred to a developing solution containing 260 μM citric acid, 66 μM formaldehyde. Silver stain development is stopped by transferring the gel to a solution containing 10% acetic acid, 45% methanol, and 45% water.

Amino Acid Composition—Amino acid analyses were done on acid hydrolysates (6 N HCl; 20–24 h) by using HPLC separation and the Pico-tag (Waters Associates, Milford, MA) procedures as previously described (19, 20). Dried hydrolysates were redissolved in 5 μl of water and derivatized with 20 μl of phenylisothiocyanate/triethylamine/ethanol (1:1:8, v/v/v) for 20 min at room temperature. Preliminary automated Edman degradations were done on an Applied Biosystems model 470A sequencer and phenylthiohydantoin amino acids were identified using a Du Pont Zorbax ODS column in a Hewlett-Packard 1084B HPLC system interfaced to a 3357 Lab Automation System as previously described (19, 20).

Radioimmunoassays—Transferrin production by Sertoli cells was assayed by a radioimmunoassay described previously (7). An aliquot of the culture media was incubated with rabbit anti-rat transferrin antibody (Cooper Biomedical, Melvern, PA) and iodinated transferrin for 1 h at 37 °C followed by a 1-h incubation with goat anti-rabbit IgG antibody (Sigma). Complexed antibody was then precipitated with polyethylene glycol (Sigma), pelleted by centrifugation, and radioactivity in the pellets determined. All data was normalized per micrograms of Sertoli cell DNA at the time of media collection and expressed as nanograms of transferrin per micrograms of DNA.

Androgen-binding protein was assayed by a radioimmunoassay described previously (11). An aliquot of the culture media was incubated with rabbit anti-rat ABP (National Hormone and Pituitary Program, National Institutes of Health, Bethesda, MD) and iodinated ABP at 4 °C for 18 h. Goat anti-rabbit IgG and polyethylene glycol were then added, and the samples were incubated an additional 18 h at 4 °C. The antibody complex was then pelleted by centrifugation and the radioactivity was determined.

DNA and Protein Assays—DNA was measured fluorometrically with ethidium bromide (21). At the end of the culture period, the media was removed, ethidium bromide buffer (EBB) (20 mM sodium chloride, 5 mM EDTA, 10 mM Tris, pH 7.5; Sigma) was added to the wells, and the cells were sonicated. An aliquot of the sonicated cell suspension was added to an equal volume of ethidium bromide solution (0.25 mM ethidium bromide, 100 units/ml heparin in EBB) and diluted 1:2 with EBB buffer and allowed to incubate at room temperature for 30 min. Fluorescent emission at 585 nm with 350 nm excitation was then monitored. A standard curve with calf thymus DNA was used to quantitate DNA levels in the culture wells. This assay has a sensitivity of approximately 0.1 μg of DNA and is linear up to 2.5 μg of DNA. Total protein concentration was measured according to the method of Bradford (22).

[1] The abbreviations used are: FSH, follicle-stimulating hormone; HPLC, high pressure liquid chromatography; PSP, peritubular cell-secreted protein; SDS, sodium dodecyl sulfate; FIRT, follicle-stimulating hormone, insulin, retinol, and testosterone; ABP, androgen-binding protein.

RESULTS

Serum-free conditioned medium was obtained from cultures of testicular peritubular cells and concentrated 100-fold by ultrafiltration with a 10,000 molecular weight exclusion limit membrane. This concentrated conditioned medium will be referred to as peritubular cell-secreted protein (PSP). Preparations of PSP generally have a protein concentration between 0.5 and 1.0 mg/ml. An ammonium sulfate precipitation is the initial step in the purification of P-Mod-S. PSP was made 70% (wt/vol) saturated with solid ammonium sulfate, agitated 18 h at 4 °C, then centrifuged at 10,000 × *g* for 30 min at 4 °C. The resulting pellet was reconstituted in a minimal volume and applied to a size exclusion HPLC column. Protein elution was monitored at 280 nm, and aliquots of individual fractions were used to treat cultured Sertoli cells (Fig. 1). Proteins eluting at a retention time between 70 and 80 min with an approximate molecular weight of 50,000 had the ability to stimulate transferrin production by Sertoli cells (Fig. 1). The peak of activity was then applied to a heparin-Sepharose affinity column. Proteins that bound to the heparin affinity column were eluted with 1 M NaCl and had the ability to stimulate transferrin production by Sertoli cells, data not shown. The proteins that bound and were eluted from the heparin affinity column were then acidified with phosphoric acid to pH 2.0 and applied to a reverse-phase (C4) HPLC column. Protein was eluted from the reverse-phase column with a 60-min linear gradient from 25 to 60% acetonitrile and monitored at 214 nm absorbance (Fig. 2). Aliquots of individual fractions were dried and used to treat cultured Sertoli cells. Two peaks of protein had the ability to stimulate transferrin production by Sertoli cells, the first having a 56-min retention time and will be referred to as P-Mod-S (A) and the second eluted with a 76-min retention time and will be referred to as P-Mod-S (B) (Fig. 2). As a final step in the purification, active fractions from the reverse-phase column shown in Fig. 2 were applied to a speed vacuum concentrator to reduce the volume 10-fold and then reapplied to the reverse-phase column. Protein was eluted with a 60-min linear gradient from 25 to 60% acetonitrile in 0.1% trifluoroacetic acid and monitored at 214 nm absorbance. P-Mod-S (A) eluted as a single peak of absorbance and activity with a 56-min retention time (Fig. 3). P-Mod-S (B) eluted as a single peak of activity with a 72-min retention time (Fig. 4). The split *bar graph* shown in Fig. 4 indicates the collection of 1.0-ml fractions. To confirm that the active peak fractions obtained were homogeneous, some samples were reapplied to a third reverse-phase column. The elution profile of the peak fractions for P-Mod-S (A) and P-Mod-S (B) are shown on the *inset graphs* of Figs. 3 and 4, respectively. A single homogeneous peak was obtained for both P-Mod-S (A) and P-Mod-S (B) with no detectable contaminant peaks (Figs. 3 and 4). These data indicate that both forms of P-Mod-S prepared were chromatographically pure.

The purity of the P-Mod-S preparations were determined with SDS-polyacrylamide gel electrophoresis and visualized with a silver stain procedure. Data presented in Fig. 5 demonstrate the purity of several different P-Mod-S preparations.

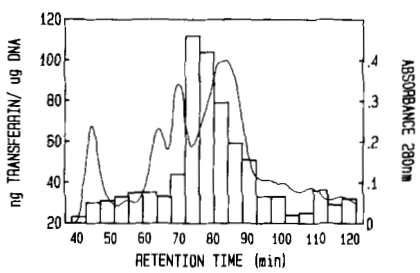

FIG. 1. **Size exclusion HPLC of peritubular cell-secreted proteins.** Protein elusion was monitored at 280 nm (*line graph*). P-Mod-S bioactivity was determined by the ability of individual fractions to stimulate transferrin production by Sertoli cells, represented as nanograms of transferrin per micrograms of Sertoli cell DNA (*bar graph*). Basal secretion in the absence of any column fraction was 30 ± 6 ng of transferrin/μg of DNA. Molecular weight standards eluted at 68 min for bovine serum albumin (68 kDa), 82 min for ovalbumin (42 kDa), and 95 min for chymotrypsinogen (25 kDa). Representative profile of nine experiments.

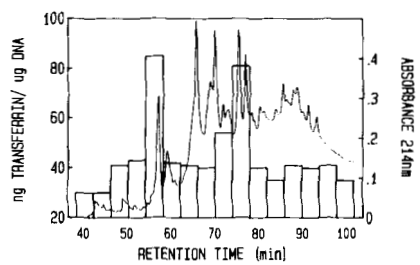

FIG. 2. **Reverse-phase HPLC of P-Mod-S obtained from a heparin-Sepharose affinity column.** Protein elution was monitored at 214 nm (*line graph*). P-Mod-S bioactivity was determined by the ability of individual fractions to stimulate transferrin production by Sertoli cells, represented as nanograms of transferrin per micrograms of Sertoli cell DNA (*bar graph*). Representative profile of nine experiments.

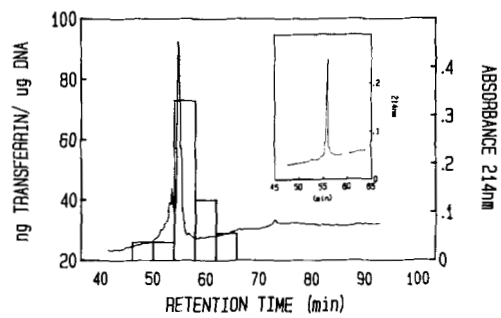

FIG. 3. **Reverse-phase HPLC of P-Mod-S (A).** Protein elution was monitored at 214 nm (*line graph*). P-Mod-S bioactivity was determined by the ability of individual fractions to stimulate transferrin production by Sertoli cells, represented as nanograms of transferrin per micrograms of Sertoli cell DNA (*bar graph*). *Inset graph*, elution profile of the active peak fraction reapplied and eluted from the reverse-phase column. Representative profile of three experiments.

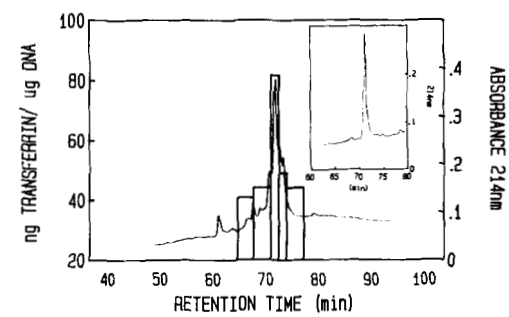

FIG. 4. **Reverse-phase HPLC of P-Mod-S (B).** Protein elution was monitored at 214 nm (*line graph*). P-Mod-S bioactivity was determined by the ability of individual fractions to stimulate transferrin production by Sertoli cells, represented as nanograms of transferrin per micrograms of Sertoli cell DNA (*bar graph*). *Inset graph*, elution profile of the active peak fraction reapplied and eluted from the reverse-phase column. Representative profile of three experiments.

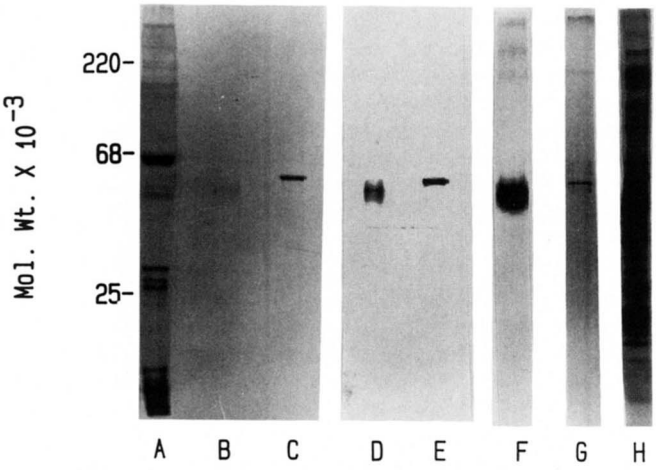

FIG. 5. **Electrophoretic analysis of peritubular cell-secreted proteins and P-Mod-S.** Proteins were detected with a silver stain procedure. Lanes are representative of peritubular cell-secreted proteins (*A*, *H*), P-Mod-S (A) (*B*, *D*, *F*), and P-Mod-S (B) (*C*, *E*, *G*). Replicate lanes are from different preparations of peritubular cell-secreted proteins and P-Mod-S.

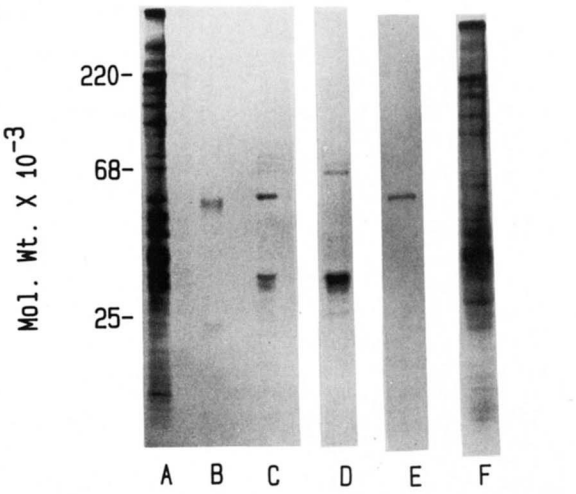

FIG. 6. **Fluorograph of an electrophoretic analysis of radiolabeled peritubular cell-secreted proteins and P-Mod-S.** Lanes are representative of peritubular cell-secreted proteins (*A*, *F*), P-Mod-S (A) (*B*), P-Mod-S (B) (*C*), P-Mod-S (B) contaminant (*D*), and P-Mod-S (B) (*E*). Replicate lanes are from different preparations.

P-Mod-S (A) was found to be a homogeneous preparation with an apparent molecular weight between 54,000 and 56,000 (Fig. 5). P-Mod-S (A) isolated from three separate PSP preparations is shown and appears as a diffuse band on the gel. P-Mod-S (B) was also found to be a single silver-stained protein with an apparent molecular weight of 59,000 (Fig. 5). Variability in the staining pattern of the bands reflect the analysis of different quantities of protein. Analysis of approximately 25–50 ng of protein is shown in *lanes B–D* and *G* of Fig. 5. An overloaded P-Mod-S (A) sample containing approximately 200 ng of protein is shown in *lane F* and demonstrates the absence of any contaminating proteins. A P-Mod-S (B) sample containing approximately 90 ng of protein is shown in *lane E* and also demonstrates the absence of contaminating proteins. The sensitivity of this silver stain procedure was found to be 0.5 ng for both bovine serum albumin and ovalbumin. Therefore, the P-Mod-S preparations obtained appear to be homogeneous at this sensitivity with the procedure utilized. Variability in the staining pattern of PSP shown in Fig. 5 reflect the loading of 100 μl of unconcentrated peritubular cell-conditioned medium in *lane A* and 500 ng of concentrated PSP in *lane H*. Results of this electrophoretic analysis indicate that a homogeneous preparation of P-Mod-S (A) and P-Mod-S (B) were obtained that are 56,000 and 59,000 molecular weight proteins, respectively.

To determine that P-Mod-S (A) and P-Mod-S (B) are actively synthesized and secreted by peritubular cells, radiolabeled secreted proteins were utilized. Peritubular cells were cultured in the presence of [^{35}S]methionine and [^{3}H]glycine, and radiolabeled secreted proteins were collected. P-Mod-S was purified from the radiolabeled proteins as described previously. Due to the low amount of radioactivity present a greater portion of the peak fractions from the second reverse-phase column were utilized which increased the possibility of the presence of contaminating proteins. The radiolabeled P-Mod-S preparations were electrophoretically analyzed and fluorographed (Fig. 6). The 56,000 P-Mod-S (A) was found to contain radioactivity as well as the 59,000 P-Mod-S (B) preparation. The radioactive proteins detected in *lanes B* and *C* of Fig. 6 were stained with the silver-staining procedure and are shown in *lanes D* and *E* of Fig. 5, respectively. The P-Mod-S (B) preparation had a contaminant of 30,000 present that eluted with a slightly greater retention time on the reverse-phase column, *lane D*, Fig. 6, and did not contain the ability to stimulate transferrin production by Sertoli cells. This contaminant appears to be a minor component of the preparation due to its lack of detection by the silver-staining procedure. A more highly purified preparation of radiolabeled P-Mod-S (B) was obtained and found not to contain any detectable contaminating radiolabeled proteins, *lane E* of Fig. 6. Results of this experiment indicate that radioactive amino acids are incorporated into the 56,000 P-Mod-S (A) and 59,000 P-Mod-S (B), which demonstrates that these proteins are actively synthesized and secreted by peritubular cells.

TABLE I

Amino acid composition of P-Mod-S

The amino acid content is presented as residues/molecule derived from the total number of calculated residues. Numbers are the mean ± S.E. for P-Mod-S (A), $n = 4$, and P-Mod-S (B), $n = 5$.

Amino acid	P-Mod-S (A)	P-Mod-S (B)
	residues/molecule	
Asx	37 ± 3	44 ± 4
Glx	34 ± 4	60 ± 2
Ser	42 ± 2	35 ± 2
Gly	46 ± 3	48 ± 2
His	11 ± 0	11 ± 1
Arg	26 ± 2	31 ± 2
Thr	30 ± 2	29 ± 2
Ala	31 ± 2	40 ± 2
Pro	43 ± 3	32 ± 2
Tyr	23 ± 2	22 ± 2
Val	32 ± 2	35 ± 2
Met	6 ± 1	9 ± 1
Ile	18 ± 2	25 ± 2
Leu	30 ± 2	49 ± 3
Phe	25 ± 2	22 ± 2
Lys	39 ± 2	50 ± 3
Residues (calculated)	512	532

The amino acid composition of P-Mod-S (A) and P-Mod-S (B) are shown in Table I. Although the compositions of many of the specific amino acids are similar between P-Mod-S (A) and P-Mod-S (B) several distinct differences were found. Examples are the proline and serine content of P-Mod-S (A) being higher than that of P-Mod-S (B), while the leucine and glutamine/glutamic content of P-Mod-S (B) is significantly higher than for P-Mod-S (A), Table I. Due to these differences any homologies between P-Mod-S (A) and

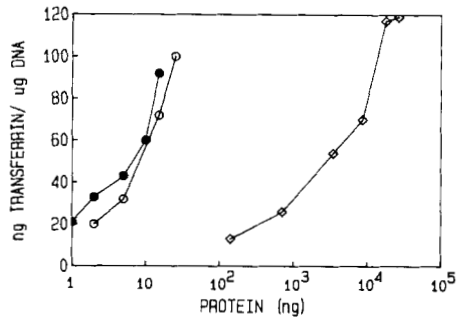

FIG. 7. **Dose response curves of peritubular cell-secreted proteins and P-Mod-S.** The effect of varying concentrations (ng/ml) of PSP (◇), P-Mod-S (A) (○), and P-Mod-S (B) (●) on transferrin production by Sertoli cells, represented as nanograms of transferrin per micrograms of Sertoli cell DNA.

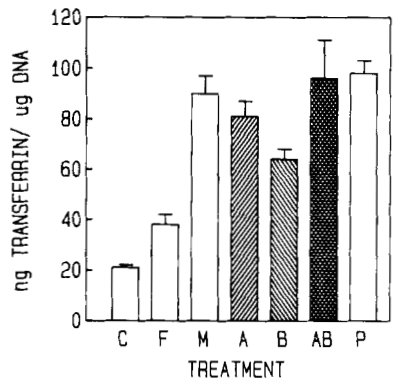

FIG. 8. **Effects of P-Mod-S and hormones on transferrin production by Sertoli cells.** Different treatments included: control (*C*), FSH (*F*), mixture of FSH, insulin, retinol, and testosterone (*M*), P-Mod-S (A) (*A*), P-Mod-S (B) (*B*), combination of P-Mod-S (A) and P-Mod-S (B) (*AB*), and peritubular cell-secreted proteins (*P*). The concentration of P-Mod-S (A) and P-Mod-S (B) utilized was between 25 and 50 ng/ml. Data is presented as nanograms of transferrin per micrograms of Sertoli cell DNA for the mean ± S.E. for $n = 9$.

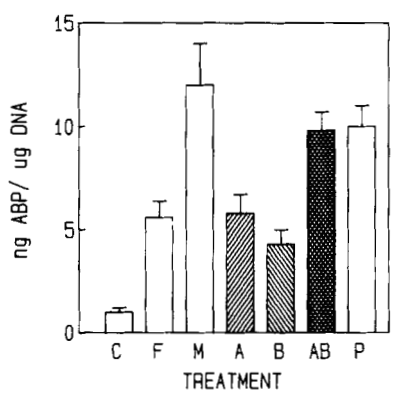

FIG. 9. **Effects of P-Mod-S and hormones on androgen-binding protein production by Sertoli cells.** Different treatments included: control (*C*), FSH (*F*), mixture of FSH, insulin, retinol and testosterone (*M*), P-Mod-S (A) (*A*), P-Mod-S (B) (*B*), combination of P-Mod-S (A) and P-Mod-S (B) (*AB*), and peritubular cell-secreted protein (*P*). The concentration of P-Mod-S (A) and P-Mod-S (B) utilized was between 25 and 50 ng/ml. Data is presented as nanograms of ABP per micrograms of Sertoli cell DNA for the mean ± S.E. for $n = 3$.

P-Mod-S (B) can not be determined from the amino acid composition data. Gas phase N-terminal sequence analysis of P-Mod-S (A) demonstrated the apparent presence of an acid-sensitive asparagine/proline bond which sporadically generated new sequences during the procedure. Therefore, reliable sequence data is not available for P-Mod-S (A) and will require larger amounts of protein. Several attempts to sequence 50–75 pmol of P-Mod-S (B) did not generate any sequence data which implies the possible presence of a blocked N-terminal amino acid. Larger amounts of protein will be needed to generate fragments of P-Mod-S (B) for sequence analysis.

The biological activity of P-Mod-S was determined by its ability to stimulate transferrin production by Sertoli cells. The effective concentrations of purified P-Mod-S and PSP are shown in Fig. 7. The concentrations required to cause a 50% maximal response for P-Mod-S (A) was 5–8 ng/ml, P-Mod-S (B) was 7–10 ng/ml, and PSP was 5–10 μg/ml. These results indicate that a greater than 1000-fold increase in the specific activity of P-Mod-S is achieved with the purification scheme utilized. Both P-Mod-S (A) and P-Mod-S (B) individually contained similar amounts of biological activity, Fig. 7. To more thoroughly assess the effects of P-Mod-S, maximal concentrations, greater than 20 ng/ml, from independent preparations of P-Mod-S (A) and P-Mod-S (B) were used to treat cultured Sertoli cells. Purified forms of P-Mod-S stimulated transferrin production by Sertoli cells approximately 80% of that obtained with PSP stimulation (Fig. 8). A combination of P-Mod-S (A) and P-Mod-S (B) did not result in an additive response but did generally increase the magnitude of the stimulation to that of PSP (Fig. 8). Whether the treatments received during the purification procedure reduced the biological activity of P-Mod-S or the combined actions of the different forms of P-Mod-S are required to get a maximal response remains to be investigated. However, results indicate that the two forms of P-Mod-S can account for essentially all the biological activity present in PSP. The ability of P-Mod-S *versus* several hormones to stimulate transferrin production by Sertoli cells is also presented in Fig. 8. As previously demonstrated FSH stimulates transferrin production approximately 2-fold while a combination of FSH, insulin, retinol, and testosterone (FIRT) is required to obtain a maximal stimulation (7). The two forms of P-Mod-S stimulated transferrin production to the same extent as FIRT (Fig. 8). P-Mod-S (A) and P-Mod-S (B) stimulated transferrin production 90 and 80%, respectively, of that obtained with FIRT stimulation. Both forms of P-Mod-S alone had a greater effect on transferrin production by Sertoli cells than FSH alone (Fig. 8). In a similar experiment using androgen-binding protein (ABP) production by Sertoli cells, a combination of P-Mod-S (A) and P-Mod-S (B) was also found to stimulate ABP production to the same extent as FIRT and PSP (Fig. 9). The individual forms of P-Mod-S stimulated ABP production similarly as FSH (Fig. 9). These results indicate that the effects of purified forms of P-Mod-S on Sertoli cell function are not restricted to a regulation of transferrin production.

DISCUSSION

Peritubular cells have previously been shown to produce a factor termed P-Mod-S that has a significant effect in modulating a number of Sertoli cell functions (9, 10). The current study was designed to purify P-Mod-S using a stimulation of transferrin production by Sertoli cells as a bio-assay for P-Mod-S. High pressure size exclusion chromatography demonstrated that under physiological conditions P-Mod-S has an apparent molecular weight of 50,000. P-Mod-S was found to have an affinity for heparin and could be separated into two forms by reverse-phase chromatography. P-Mod-S (A) was the least hydrophobic bioactive protein and was found to

have a molecular weight between 54,000 and 56,000 using SDS-gel electrophoresis. The diffuse band of P-Mod-S (A) on the SDS gel implies that P-Mod-S (A) may be a glycoprotein and/or have charge heterogeneity. P-Mod-S (A) appeared chromatographically pure on a reverse-phase column and electrophoretically pure as detected by a silver-staining procedure. P-Mod-S (B) was the more hydrophobic bioactive protein and was found to have a molecular weight of 59,000 using SDS-gel electrophoresis. Both reverse-phase chromatography and electrophoretic analysis with a silver-staining procedure demonstrated that a pure preparation of P-Mod-S (B) was obtained. P-Mod-S was isolated from peritubular cell-radiolabeled secreted proteins and both P-Mod-S (A) and P-Mod-S (B) contained radioactivity. One P-Mod-S (B) preparation was found to contain a contaminant with a 30,000 molecular weight that was radioactive but could not be detected with the silver stain procedure. Therefore, this contaminant appeared to be a minor component of the preparation. This 30,000 molecular weight protein was found to be due to an overlapping protein peak that was more hydrophobic than P-Mod-S (B) and when purified was found not to contain biological activity. In support of this observation a more highly purified preparation of radiolabeled P-Mod-S (B) did not contain any contaminants. The observation that P-Mod-S (A) and P-Mod-S (B) contain radioactivity provides direct evidence that peritubular cells actively synthesize and secrete both forms of P-Mod-S and that the activity is not a contaminant from a previous treatment of the peritubular cell cultures.

Due to the differences in molecular weight between P-Mod-S (A) and P-Mod-S (B) the possibility exists that the 59-kDa P-Mod-S (B) may be a precursor or larger form of P-Mod-S (A). Although protease inhibitors were present throughout the isolation procedure, P-Mod-S (A) may also be a proteolitically degraded form of P-Mod-S (B). The amino acid composition of the two forms of P-Mod-S have similarities, however, the total composition does not support the concept of interconversion of the two forms, particularly due to the high proline and serine content of P-Mod-S (A). More definitive information concerning the relationship of the two forms of P-Mod-S will require amino acid sequencing, antibody production, and preparation of molecular probes for the proteins. Preliminary attempts on N-terminal sequencing of the proteins revealed that P-Mod-S (A) appears to be an acid-sensitive protein, probably due to the presence of a sensitive proline/asparagine bond, and P-Mod-S (B) appears to have a blocked N-terminal amino acid. Further studies will require larger amounts of protein for analysis. The typical yields of the individual forms of P-Mod-S purified from 5 liters of peritubular cell-conditioned medium ranged from 3–8 μg of protein. Currently, large scale isolations are in progress to increase the yields 5- to 10-fold for further studies.

Determination of the biological activity of P-Mod-S (A) and P-Mod-S (B) demonstrated that a greater than 1000-fold increase in specific activity was achieved with the purification procedure utilized. Both forms of P-Mod-S had similar amounts of bioactivity and required between 1 and 10 ng/ml for stimulation of transferrin production. The combined actions of P-Mod-S (A) and P-Mod-S (B) was found to increase the magnitude of stimulation slightly but was not generally an additive response for transferrin production. This result supports the possibility that these two forms of P-Mod-S may be related. However, the current study does not provide conclusive information regarding the relatedness of the different forms of P-Mod-S and further experiments are required. The magnitude of the response of Sertoli cells to P-Mod-S was similar to that of a combination of FSH, insulin, retinol, and testosterone, which results in a maximal stimulation of several cellular functions (7, 23). These results indicate that P-Mod-S appears to have a greater ability to stimulate Sertoli cell function than any individual regulatory agent previously known to influence Sertoli cell function, including FSH. A crude preparation of P-Mod-S in PSP has been shown to maximally stimulate a number of Sertoli cell functions including total protein production, transferrin and ABP production, and the production of all major radiolabeled secreted proteins (9, 10). ABP production by Sertoli cells was examined to investigate whether the purified forms of P-Mod-S also have these wide spread effects on Sertoli cell function. Both forms of P-Mod-S were found to maximally stimulate ABP production in a manner similar to the combination of FSH, insulin, retinol, and testosterone. Therefore, the purified forms of P-Mod-S can account for essentially all the activities detected in total peritubular cell-secreted protein using two major markers of Sertoli cell function, transferrin and ABP production.

Sertoli cells play an integral role in the maintenance and control of germinal cell development. Therefore, regulatory agents which modulate Sertoli cell function will indirectly influence the process of spermatogenesis. Because P-Mod-S has a dramatic effect in modulating Sertoli cell functions, P-Mod-S is postulated to have an important role in regulating testicular function. Androgens are required for testicular function and act locally on the somatic cells of the seminiferous tubules (24). Peritubular cells contain a high percentage of the androgen receptors in the tubule (25, 26) and may be a site of androgen action (27). Observations presented in a previous report indicate that androgens may regulate P-Mod-S production by peritubular cells (9). An interaction between the cell types in the testis is proposed in which Leydig cells produce androgens which can act on peritubular cells to maintain or stimulate the production of P-Mod-S which can then act on Sertoli cells to modulate functions important for germinal cell development. This peritubular cell-Sertoli cell interaction mediated via P-Mod-S is postulated to provide a mode of androgen action in the testis. In support of this hypothesis is the observation that the presence of peritubular cells in a Sertoli cell culture system augments the effects of androgens on Sertoli cell function, presumably through the actions of P-Mod-S (11). Future studies will require a quantitative assessment of the effects of androgens on P-Mod-S production to provide insight into this potentially important cell-cell interaction.

The interactions between peritubular cells and Sertoli cells provide an example of mesenchymal-epithelial cell interactions. Previous investigations using an embryological approach of mesenchymal-epithelial cell interactions has resulted in the hypothesis that mesenchymal cells produce inducer substances, often under steroid control, which regulate the functions and differentiation of the adjacent epithelial cells (for review, see Ref. 14). The interaction between peritubular cells and Sertoli cells mediated by P-Mod-S provides direct biochemical evidence for this hypothesis. Further investigation into P-Mod-S and peritubular cell-Sertoli cell interactions will provide insight into general mesenchymal-epithelial cell interactions. Whether P-Mod-S is a tissue-specific mesenchymal inducer substance or a more general mediator of cell-cell interactions in a number of tissues remains to be determined. The possibility that P-Mod-S may be a general mediator of androgen actions will also be of interest for further investigations.

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