Sertoli Cells in Culture Release a Pregnancy Specific β1 Glycoprotein-like Substance (44080)

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Abstract. **The possibility that pregnancy-specific β1 glycoprotein (PSG) is released by Sertoli cells was investigated by using reverse hemolytic plaque assays which enable the visualization of release from individual cells in culture. We found that the proportions of cells releasing PSG increased gradually in 3-day old cultures prepared from animals of increasing age (10-, 20-, and 40-day old animals). The gradual appearance of PSG-releasing cells during this period differed markedly from that of transferrin (TF)-releasing cells, suggesting that the age-related development of PSG-releasing cells is regulated in a specific manner. PSG cells were also found in Sertoli cell cultures prepared from stage-associated seminiferous tubule segments of adult rat testes. The percentages of PSG plaque-forming cells differed from one stage-associated culture to another with maximal proportions associated with stages III–V and XIII, and minimal proportions found in stages VII, and IX–XI. The abundance of Sertoli cells that released PSG from stage to stage differed markedly from those that released TF indicating that modulatory processes specific for PSG are also present in the adult testis. Finally, PSG cells were also identified immunocytochemically in cultures prepared from staged tubule segments. The proportion of PSG staining cells from stage to stage were found to be virtually indistinguishable from those identified with plaque assays. When taken together, these results show clearly that Sertoli cells in culture release a PSG-like molecule. Moreover, this release appears to be controlled in an age-related and stage-dependent manner, suggesting that Sertoli cells may be central to PSG function in the testis.** [P.S.E.B.M. 1997, Vol 214]

Pregnancy-specific β1 glycoprotein (PSG) was isolated initially from human placental extracts and sera during pregnancy. This molecule was found to have a high degree of molecular heterogeneity (1, 2) and to be synthesized in large quantities by syncytiotrophoblasts of the placenta (3–5). Since its initial identification and isolation, PSG expression has been found in a variety of different cell and tissues. These include normal cells such as cultured fibroblasts and brain cells (6, 7) as well as abnormal cells such as ovarian adenocarcinoma, malignant glial, HeLa, and placental tumor cells (7–10). Many of these sites of PSG expression are associated with growth processes or rapidly changing cell architecture raising the possibility that other locations with similar characteristics may be associated with PSG.

Recent evidence indicates that one of these, the testis, which is in a dynamic state of germ cell growth and development, may be an important site of PSG synthesis and release. The expression of a PSG-like substance has been identified in both human (11) and rat testis tissue (10, 12, 13). It is not clear, however, which testicular cells are associated with this molecule. In the rat, Ogilvie *et al.* (12) demonstrated the presence of PSG immunoreactive material in the interstitial compartment as well as certain cells within the seminiferous tubule region, thought to be mainly germ cells. Later studies by Richardson and co-workers (13) identified mRNA that hybridized to a cDNA probe for PSG localized mainly to the interstitium with only very diffuse

This work was supported in part by National Institutes of Health Grant HD-25090 from the NICHHD.

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Received May 3, 1996. [P.S.E.B.M. 1997, Vol 214]
Accepted September 10, 1996.

0037-9727/97/2142-0139$10.50/0

signal present within the tubule epithelium. These studies clearly demonstrate an interstitial site of expression of PSG in the testis. However, it still remains unclear whether PSG is present within the tubule and if so whether it is associated with germ cells and/or another cell type such as Sertoli cells. The inability to observe a strong localized in situ signal for PSG mRNA within the tubule may not be due to an absence of message, but rather to its presence in a cell with diffuse cytoplasmic characteristics. Sertoli cells possess such a diffuse cytoplasm which has been shown to influence the distribution and intensity of certain messages detected by *in situ* hybridization (14). Moreover, because of the extensive role of Sertoli cells in the establishment and maintenance of the seminiferous tubular environment, it is quite possible that Sertoli cells may synthesize PSG or transfer this molecule from the interstitium into the seminiferous tubular compartment. This would be quite consistent with normal Sertoli cell function (for reviews see Refs. 15–17). In order to address the possibility of whether Sertoli cells are associated with a PSG-like substance in this study, we subjected cultured cells isolated from developing and mature animals to reverse hemolytic plaque assays and immunocytochemistry for PSG. This approach provides a sensitive and specific means of directly determining whether the release and storage of a PSG-like product is associated with individual Sertoli cells. Our results clearly demonstrate the presence of PSG plaque-forming and staining cells in each of these cultures, suggesting strongly that Sertoli cells may function in both developing and adult animals to store and release a PSG-like substance.

Material and Methods

Animals and Cell Culture. Testes obtained from 10-, 20-, and 40-day-old rats (Holtzman Laboratory Animals, Madison, WI) were dispersed using a modified version of the procedure of Rich *et al.* (18) that was described previously (19). In short, testes were decapsulated and placed into minimum essential medium (MEM; Gibco, Grand Island, NY) containing collagenase-dispase (0.03%; Boehringer-Mannheim Biochemicals, Indianapolis, IN) and hyaluronidase (0.05%, type I-S; Sigma Chemical Co., St. Louis, MO). The solution was first incubated for 30 min with constant stirring and then transferred to a 50-ml sterile tube and allowed to settle. The supernate which contained mainly interstitial cells was discarded. The precipitated seminiferous tubules were minced and placed into another enzyme solution (0.045% collagenase-dispase and 0.05% hyaluronidase) for 20–40 min. Tissue from 10- and 20-day-old rats was dispersed sufficiently within 20–30 min. However, a period of 40 min was necessary to disperse tissue from 40-day-old animals. After enzyme exposure, the cell suspension was placed in a 50-ml centrifuge tube and allowed to incubate for 20–30 min. During this period, Sertoli cell clumps settled to the bottom of the tube. The supernate was discarded and the clump resuspended in MEM and allowed to resettle. This step was repeated until no cells remained in the supernate. Sertoli cell aggregates were then passed repeatedly into and out of a flame-polished, siliconized Pasteur pipette, which reduced the size of the aggregates. The cell suspension was then filtered through a 25-μm Nitex screen (Tetco, Lancaster, NY) and resuspended in culture medium consisting of Dulbecco's modified eagle's medium (DMEM) and Ham's F-12 that contained 4.0% fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml), all from Gibco. The cells were placed into 35-mm petri dishes (Costar Corp., Cambridge, MA) at a concentration of approximately 350,000 aggregates/dish and incubated at 34.5°C in a water-saturated atmosphere of 5.0% CO_2/95% air. During the first day of culture, the Sertoli cells attached and spread on the floor of the culture dishes resulting in approximately 0.75–1.50 × 10^6 Sertoli cells/dish. On the second and third days of culture, the medium was changed, and the unattached germ cells and debris were removed.

Sertoli cells from stage-specific seminiferous tubule segments of adult rats were obtained as reported previously (20). Briefly, testes from mature rats (>70 days; Holtzman) were placed into a solution of collagenase/dispase and hyaluronidase as described above and gently shaken for 60–90 min. After this period, the tubules were rinsed with MEM transferred to a large glass petri dish and subjected to a microdissection procedure using transilluminated light as described by Parvinen and Ruokonen (21). Using distinct shading patterns, we obtained tubule segments corresponding to III–V, VII, IX–XI, and XIII of the seminiferous epithelial cycle. Each staged segment group was placed into an enzyme solution containing 0.02% collagenase III (Worthington Biochemical Corp., Freehold, NJ) and 0.07% hyaluronidase in MEM at 34.5°C and stirred gently for 30 min. Fragments resulting from this procedure were passed repeatedly through a flame-polished Pasteur pipette and rinsed with fresh MEM. Cells were then resuspended in culture medium and approximately 2 ml (4.0 × 10^6 cells/ml) were placed into each 35-mm tissue culture dish. The dishes were incubated for 48 hr at 34.5°C in a water-saturated atmosphere of 5% CO_2/95% air. During this period, the Sertoli cells attached to the floor of the dish. Germ cell and debris which did not attach were removed by washing the cultures vigorously three times with MEM after 48 hr of culture. The cultures were allowed to incubate for an additional 24 hr and then were subjected to plaque assays or immunocytochemistry.

Reverse Hemolytic Plaque Assay for PSG or Transferrin. Reverse hemolytic plaque assays were performed as previously reported (22–24) for pituitary hormones or transferrin (TF) with modifications made to enable detection of PSG. On the day of an experiment, cultures obtained from immature animals or tubule segments from adults were exposed to a dilute solution of trypsin (0.025% in MEM) for 5 min. The cells were detached and monodispersed by directing the solution onto the monolayer

and into and out of a Pasteur pipette. The cells were then washed and diluted in assay medium (Dulbecco's modified eagle's medium with 0.1% BSA and antibiotics). An aliquot of this preparation (500,000 cells/ml) was mixed with an equal volume of protein-A–coated bovine red blood cells (9.0% suspension) and infused by capillary action into poly-*l*-lysine–coated Cunningham chambers. The cells settled and attached to the floor of the chambers forming monolayers. The chambers were washed with assay medium to remove unattached cells and then filled with a solution containing either TF or PSG antiserum. Transferrin antiserum (rabbit anti-rat; 4.0 mg/ml antibody protein; U.S. Biochemical Corp., Cleveland, OH) was diluted at 1/80 or PSG antiserum (rabbit anti-human; Dakopatts, Carpinteria, CA) was diluted at 1/250 with assay medium. The chambers were incubated for an 8-hr period, which was found to be maximal for plaque detection. After incubation, a solution of complement (1/40 or 1/20 dilution of guinea pig serum [Gibco] in assay medium for TF and PSG assays, respectively) was infused to initiate plaque formation. The reaction was terminated after 1 hr by infusion of a fixative (1.8% glutaraldehyde in isotonic saline). A typical PSG plaque-forming cell is presented in Figure 1. A plaque former is identified by the presence of a surrounding area of lysed red blood cells. For quantification, cells were stained with toluidine blue and the proportions of plaque-forming cells determined by evaluation of at least three different microscopic fields that were selected randomly. Specific controls were performed both for TF and PSG plaque assays. As reported previously, we found that preabsorption of 200 μl of a working dilution of TF antiserum (1/80) with purified rat TF (1 μg, Sigma) for 2 hr at 34.5°C completely abolished plaque formation. For PSG, it was determined that preabsorption of 200 μl of a working dilution of PSG antiserum (1/250) with purified human PSG (8 μg; The Binding Site, Limited, Birmingham, England) for the same period completely eliminated plaque formation. Also, our removal of antibody or complement for either assay abolished the formation of plaques.

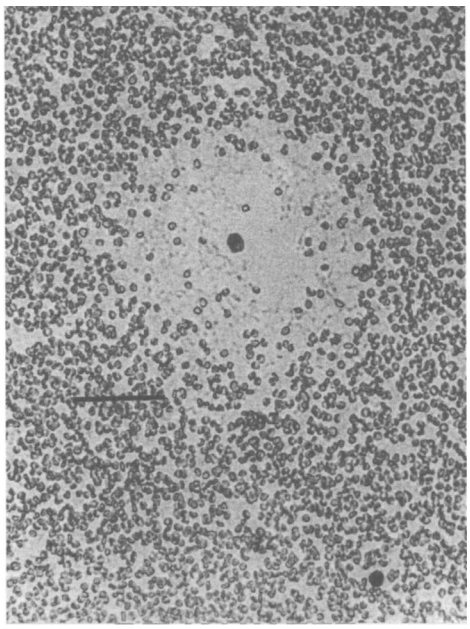

Figure 1. Example of a PSG-secreting Sertoli cell. PSG release is identified by a plaque, area of lysed red blood cells surrounding the Sertoli cell. A non–plaque former is also present in the lower right hand corner of the field. This micrograph was obtained using Nomarski optics. Bar, 50 μm.

Immunocytochemistry for PSG. Immunocytochemistry was performed as reported previously (24) except that modifications were made for PSG detection. Briefly, adult Sertoli cells were recovered from culture, placed on poly-*l*-lysine–treated microscope slides and incubated at 34.5°C for 1 hr. The slides were removed from the incubator and subjected to a solution of 9:1 B-5 fixative (0.25 *M* mercuric chloride and 0.2 *M* sodium acetate) and formaldehyde for 10 min followed by consecutive 1-min washes with 95% ethanol, double-distilled water, and Lugol's iodine (0.08 *M* iodine and 0.12 *M* sodium thiosulfate). The slides were then submerged in Tris-saline (0.05 *M* Tris in 0.15 *M* NaCl, pH 7.6) for 15 min. Endogenous peroxidase activity was removed by incubating the slides with 3% hydrogen peroxide for 5 min. PSG was localized by using the avidin-biotin peroxidase complex method. This was accomplished by using the Vectastain ABC Kit (rabbit immunoglobulin G/PK 4001; Vector, Burlingame, CA) and anti PSG primary antiserum, the same as described above. The cells were preincubated with 30% normal goat serum for 20 min, followed by a 2-hr incubation with PSG antiserum (1:500). Biotinylated antirabbit immunoglobulin G was then added and incubated for 30 min. Finally, the slides were exposed to avidin-biotin peroxidase complex reagent. Specific reaction product was localized with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. This appeared as a deep brown stain found throughout the cell cytoplasm. The proportions of staining cells were quantified by evaluation of at least three different areas of each slide. Cell specific staining was absent when primary antiserum was replaced with normal rabbit serum or any reagent in the kit was replaced with Tris saline.

Alkaline Phosphatase Histochemistry for Myoid Cells. Myoid cell contamination was monitored by the procedures of Palombi and DiCarlo (25). Cultured cells were placed on poly-*l*-lysine–treated glass slides and allowed to attach for 1 hr at 34.5°C. The slides were air-dried, immersed in ice cold acetone-ethanol fixative (1:1 v/v for 10 min at −20°C and transferred to 0.1 *M* phosphate buffered saline. A solution of 0.5 ng/ml Fast Blue RR (Sigma) in Tris-HCl buffer (pH 8.6) and 40 μg/ml of α-napthal phosphate (0.25% solution, Sigma) was added and the slides incubated for 30 min. Slides were then rinsed and mounted with Crystal Mount (Fisher Scientific Co, Pittsburgh, PA). The cells staining for alkaline phosphatase contained a blue reaction product. With the exception of cultures obtained from Day 10 animals which contained 10.9% ± 0.7%

stained cells (mean ± SEM, n = 5 separate experiments), the proportion of cells that were positive for alkaline phosphatase were always less than 3.0%.

Statistics. Student's *t* tests were employed to detect differences between treatment groups when single comparisons were made. Analysis of variance followed by Tukey's multiple range test (26) was used to assess statistical significance of multiple comparisons.

Results

The proportions of PSG plaque-forming cells in cultures prepared from Day 10, Day 20, and Day 40 testes are presented in Figure 2. As shown, the percentages of cells that released PSG increased gradually in cultures prepared from animals of increasing age. This pattern was different for TF as illustrated in Figure 3. With TF, the proportions of plaque formers increased markedly between cultures from Day 10 and Day 20 animals. However, the percentages of TF secretors did not increase additionally even in cultures prepared from animals much more advanced in age (40 days old), suggesting that the proportions of TF secretors had reached a maximum in these populations by 20 days of age.

In order to determine whether adult testes also contain cells that release PSG, we subjected stage-associated Sertoli-cell cultures to plaque assays for PSG. We found that PSG-releasing cells were present in each of the stage-associated cultures evaluated, but differed from stage to stage (Fig. 4, top panel). The maximal proportions of Sertoli cells that released PSG were identified in cultures prepared from staged III–V and XIII cultures. Much smaller percentages of PSG-related cells were observed in cultures from

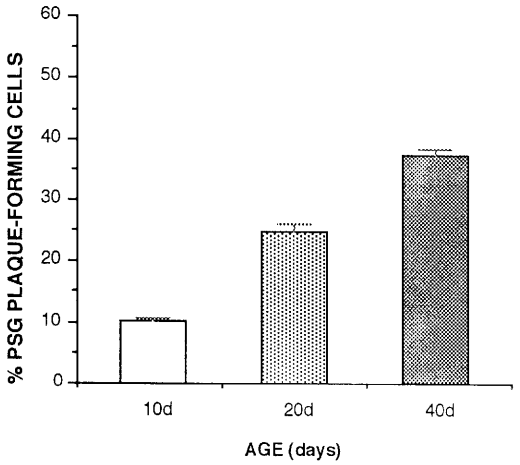

Figure 2. The percentages of PSG plaque-forming cells in Sertoli cell cultures from animals of various ages. Testes were obtained from animals at 10, 20, and 40 days of age and dispersed with enzymes to recover Sertoli cells. The cells were then subjected to plaque assays for PSG. These results are the mean and SEM of determinations made on cells from three testes dispersions for 10-day-old rats, and four testes dispersions for 20- and 40-day-old rats. At least 400 cells were counted (200 cells/plaque assay slide) for each assay.

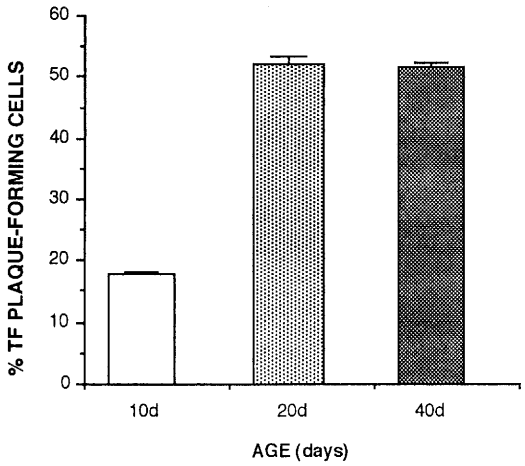

Figure 3. The proportions of TF plaque formers in Sertoli cell cultures from animals of different ages. Cells were obtained from testes of animals sacrificed at 10, 20, and 40 days of age, cultured, and then subjected to plaque assays for TF. Shown here are the results (mean ± SEM) of plaque assays performed on testis cells obtained from three different groups of 10-day-old animals and four different groups of 20- and 40-day-old animals.

staged VII and IX–XI segments. As illustrated in Figure 4 (bottom panel), the stage-dependent abundance of these cells differed markedly from those observed to secrete TF. The highest proportions of TF-releasing cells were identified in cultures from staged IX–XI and XIII segments while the lowest proportions of these secretors were found in cultures from stages III–V and VII.

Immunocytochemistry for PSG was performed on these staged cultures to determine whether cells were present that contained, but did not release, PSG. As before, PSG cells were identified in each stage-associated culture evaluated. In fact, we found that the proportions of Sertoli cells in these cultures that stained for PSG (Table I) were virtually identical to those that formed PSG plaques, indicating that we had identified most, if not all, of the PSG cells in these populations.

Discussion

Our results demonstrate clearly that a PSG-like substance is released from cultured Sertoli cells obtained during testicular development. These cells increased with the advancing age of the animal, reaching a maximum by at least 40 days of age. The pattern of PSG-releasing cells differs from that of TF cells, which appear to reach maximal proportions at a much younger age. This would indicate that the gradual PSG cell increase is not due mainly to a general secretory cell expansion but results from a specific enhancement of the population of cells that release this PSG-like substance. Moreover, it appears that most if not all of the PSG-releasing cells are Sertoli cells, because contaminating germ and myoid cells have been removed during the acquisition or culture procedures, at least for culture from animals older than 10 days (18–20). For cultures obtained from Day

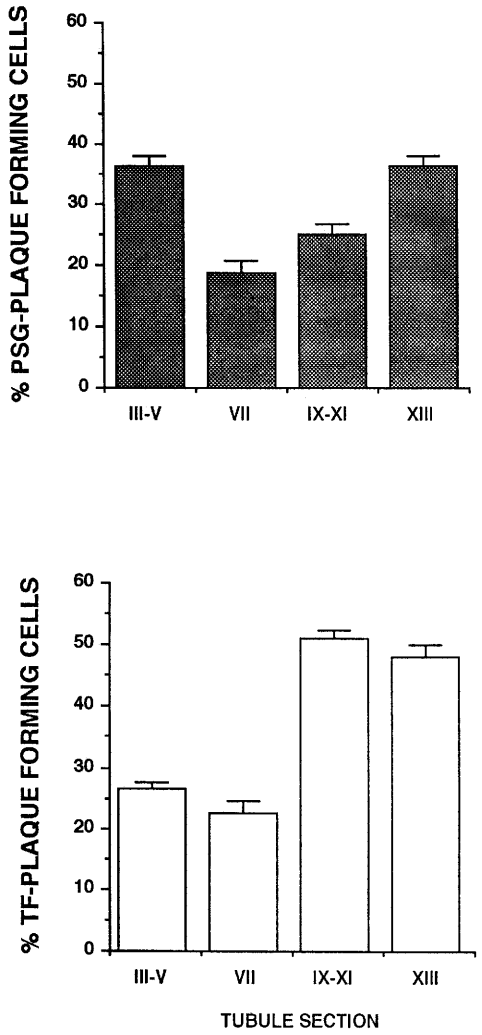

Figure 4. The percentages of plaque-forming cells in cultures prepared from different staged segments of the seminiferous epithelium. Seminiferous tubule segments corresponding to stage III–V, VII, IX–XI, and XIII were recovered by microdissection using transilluminated light, dispersed with enzymes, and placed into culture. After 3 days, plaque assays were performed on cells from each segment. Shown here are the results (mean ± SEM, $n = 5$ separate experiments) from plaque assays for PSG (top panel) and TF (bottom panel). For each experiment, determinations were made on at least 400 cells (200 per slide) for every staged segment.

10 animals, the possibility cannot be eliminated that myoid cells may contribute to PSG release because a portion of cells in these cultures were identified as myoid cells (19). Our findings of age-related changes in PSG expression in these cultures are consistent with reports of age-related shifts in PSG message abundance in several testis regions during development (13). Although most of these changes in hybridization was localized to interstitial elements, diffuse signal above background was also observed to change with age within seminiferous tubules. In light of our results, it is quite possible that some or all of this intratubular signal could have been associated with Sertoli cells.

Our results further reveal that PSG is released from Sertoli cells obtained from adult rats. In fact, the proportions

Table I. Percentages of Cells That Stained for PSG in Cultures from Stage-Associated Segments

Tubule segments			
III–V	VII	IX–XI	XIII
39.9 ± 1.2	25.9 ± 2.2	29.3 ± 1.5	41.1 ± 1.3

Note. Presented are the mean ± SEM of three separate experiments. Cell obtained from different staged segments were cultured for 72 hr, attached to poly-*l*-lysine–coated glass microscope slides, and then subjected to immunocytochemistry for PSG. Within each experiment, the proportions of staining cells were quantified by counting at least 200 cells/slide on duplicate slides for each staged segment.

of PSG cells differ from one stage-associated segment to another. The largest percentages of PSG-releasing cells were found either early or late in the cycle (stages III–V and XIII), while much smaller proportions were associated with stages VII and IX–XI more toward the middle of the cycle. The observation that the percentages of cells detected by plaque assay and immunocytochemistry were virtually identical suggests strongly that the differences identified in PSG cell proportions between segments were probably not due to an inability to detect cells that contained, but did not secrete, PSG. Most if not all cells present in these cultures are probably Sertoli cells, because myoid or germ cells, the major potential contaminants of cultures prepared from seminiferous tubular segments are in very small numbers or absent (20). Thus, the presence of these PSG cell differences from segment to segment suggests that the storage and release of the molecule in Sertoli cells is highly regulated. Moreover, this process appears to be specific for PSG. Although the proportions of TF cells also change from one stage-associated segment to another, as shown here and elsewhere (20, 27), the TF pattern is different from that of PSG. When taken together, it appears that not only do Sertoli cells from adult rats release a PSG-like substance but this release is dependent on the stage of the seminiferous epithelial cycle.

The role of PSG in testis function remains obscure. To date, PSG mRNA has been associated mainly with Leydig cells and myoid cells (13) and immunoreactive PSG has been identified in Leydig cells and germ cells (12). Our studies suggest that PSG is also released from Sertoli cells. The association of PSG with such differing cell types suggests that PSG or related molecules may have more than one function in the testis. For example, it has been suggested that PSG may contribute to the reduced immune responsiveness found in testis tissue (12, 13). Placental PSG was observed to reduce markedly human lymphocyte proliferation *in vitro* (28), indicating that this molecule has immunosuppressive properties. Alternatively, PSG alone or in combination with other molecules may act to enable communication from one part of testis to another. The observations discussed above of either immunoreactive material or mRNA in the interstitium and the seminiferous epithelium suggest that both tubular and interstitial compartments are influenced by PSG. Our findings of an association of PSG

with Sertoli cells strengthen the possibility that this influence may involve some type of compartmental interaction. It is well recognized that most if not all of the components that reach the tubular compartment must pass through or be synthesized by Sertoli cells (for reviews see Ref. 15–17). The stage-related pattern of PSG storage and release found in this study clearly demonstrates the ability of Sertoli cells to handle and export PSG in a specific manner. This PSG may originate in the interstitium and be taken up and transferred by Sertoli cells or may be synthesized in the Sertoli cell cytoplasm for release to other cell types. Either process may be important in communicating from one part of the testis to another. Finally, as suggested by Richardson *et al.* (13) for the interstitium, PSG may also function within the seminiferous epithelium to promote proper cell-to-cell interaction. A molecule with sequence homology to PSG, carcinoembryonic antigen (CEA), has been shown to function in aggregation of cultured colon adenocarcinoma cells and in sorting of cells in heterogeneous populations (29). As with CEA in tumor cell populations, PSG that would be associated with the surface of specific Sertoli and germ cells may help to form the proper cell-to-cell contacts necessary for portions of the spermatogenic process to proceed. In light of observations that late spermatids stain for PSG (12), it would be reasonable to suggest that such a process may occur in the latter stages of germ cell development. Benchimol and co-workers (29) first proposed such a cell-to-cell contact role for CEA in the rapidly changing architecture of the interstitial epithelium and suggested that it may work in concert with other homotypic adhesion molecules such as cadherin. Cadherins expressed by both Sertoli and germ cells (30) as well as other molecules with cell adhesion properties (31) have been identified in the testis, and these, in conjunction with PSG, may contribute to the dynamic cellular rearrangement that is one of the most distinctive characteristics of the seminiferous epithelium. When taken together, it appears that this PSG-like molecule has the potential to influence multiple facets of testis function. Extensive work will be needed to determine whether these or other roles can be attributed to this substance.

In summary, our results clearly demonstrate that Sertoli cells release a PSG-like molecule. This release appears to be controlled in an age-related and a stage-dependent manner. Our finding of PSG release from a component within the seminiferous tubule not only expands the possible ways in which a PSG-like molecule may act in the testis but also increases the likelihood that Sertoli cells may play a central role in one or more of these actions.

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