Sertolin Is a Novel Gene Marker of Cell-Cell Interactions in the Rat Testis*

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A novel testicular protein designated sertolin was cloned. The full-length sertolin cDNA consists of 853 base pairs with an open reading frame of 381 base pairs coding for a 127-amino acid polypeptide that shares limited identities with antaxin/josephin and thrombospondin proteins. Sertolin (calculated molecular mass, 13,759 daltons) has two mRNA transcripts of 2.3 and 1 kilobase. A 22-amino acid peptide based on the deduced amino acid sequence of sertolin (NH2-KKEHFNLFKAASVSHLVQVPQ) was synthesized and used for polyclonal antibody production. Immunoblot analysis detected a 17-kDa immunoreactive band in the Sertoli cell cytosol. Using Sertoli-germ cell cocultures, sertolin expression was found to be reduced by as much as 5-fold at the time when germ cells attach onto Sertoli cells but preceding the establishment of specialized inter-Sertoli-germ cell junctions. Neither FSH nor 17β-hydroxy-5α-androstane-3-one was able to affect sertolin expression, whereas estradiol-17β and progesterone induced a significant increase in Sertoli cell sertolin expression in vitro. In addition, interleukin-1α, a germ cell-derived cytokine, was also able to elicit a transient but significant increase in Sertoli cell sertolin expression. Sertolin expression was also shown to increase with testicular development and is likely to be associated with the onset of spermatogenesis. In addition, sertolin expression increased in the testis when generalized inflammation was induced in adult rats by injection of fermented yeast. These results show that sertolin will be useful in characterizing cell-cell interactions in the testis.

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Apart from the numerous morphological and molecular changes that take place in developing germ cells during spermatogenesis, these cells also migrate from the basal to the adluminal compartment, where fully developed spermatids are released into the tubular lumen at spermiation (1, 2). During the process of germ cell movement, it is envisioned that specialized inter-Sertoli and Sertoli-germ cell junctions must be intermittently disassembled and reassembled in a highly organized manner. As such, germ cell movement must consist of intermittent phases of junction disassembly and reassembly. These events probably also require the active participation of several proteases, protease inhibitors, junctional complex components, and signaling molecules that are found in the testis (3, 4). However, the intricate cascade(s) of events underlying spermatogenesis with respect to germ cell migration has not been elucidated. Recent studies from this laboratory have demonstrated that when germ cells consisting largely of spermatogonia and spermatocytes are cocultured with Sertoli cells in vitro for a short period of time prior to the establishment of specialized intercellular junctions, there are changes in the expression of several proteases, protease inhibitors (5, 6), and cell adhesion molecules (7) showing that germ cell attachment to Sertoli cells with the eventual establishment of specialized cell junctions consists of a series of dynamic biochemical events. A better comprehension of how germ cells interact with Sertoli cells in vitro could be instrumental in interrupting or facilitating the migration of developing germ cells from the basal to adluminal compartment, thereby disrupting male fertility, although many investigators have focused and still continue to focus primarily on understanding how to disrupt the delicate hormonal balance between the pituitary and testis as a means of fertility control. One reason for this lack of knowledge in understanding the mechanism(s) of germ cell movement in the seminiferous epithelium is the shortage of available markers that can be used to examine Sertoli-germ cell interactions in the testis. Moreover, many of the biomolecules that participate in the events of germ cell movement and cell-cell interactions are not known. Therefore, we have found it necessary to identify such molecules as part of an attempt to study the biology of Sertoli-germ cell interactions. In the present study, we have utilized the mRNA differential display technique (8–10) in conjunction with PCR1 to identify and clone a new Sertoli cell gene product designated sertolin. Much of the data presented in this paper rely on the relative levels of mRNA detected by RT-PCR, which is necessary due to the low level of expression of sertolin. We, however, have used an antibody prepared against a 22-amino acid peptide specific to sertolin to verify some of our observations. Preliminary studies illustrate that sertolin is a potential candidate that can be used to probe Sertoli-germ cell interactions particularly at the time preceding the establishment of specialized Sertoli-germ cell junctions.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank†‡ EBI Data Bank with accession number(s) AF107727.

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‡ The abbreviations used are: PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; DMEF, Dulbecco’s modified Eagle’s medium; GCCM, germ cell-conditioned medium; SCCM, Sertoli cell-conditioned medium; HPLC, high pressure liquid chromatography; IL, interleukin; bFGF, basic fibroblast growth factor; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; INF, interferon.

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**EXPERIMENTAL PROCEDURES**

**Animals**

Male or female Harlan Sprague-Dawley rats of 250–300 g, body weight, and male pups at 20 or 27 days of age were obtained (Charles River Laboratories, Kingston, MA). Rats were killed by CO2 asphyxiation. For testicular cell cultures, testes were removed immediately for the isolation of cells. For RNA extraction, organs were removed, frozen in liquid nitrogen immediately, and stored at −80 °C until use. The use of animals and reagents was approved by the Rockefeller University Animal Care and Use Committee with Protocol Numbers 95129-R1, 91353-R1, 91353-R2, and 97117.

**Preparation of Testicular Cell Cultures**

**High and Low Density Sertoli Cell Cultures—**Primary Sertoli cell cultures were prepared from 20-day-old male rats by sequential enzymatic treatments as described previously (5, 11–13). For high density Sertoli cell cultures to allow the formation of specialized occluding, anchoring, and communicating junctions, isolated cells were plated on Matrigel™ (Collaborative Biochemical Products, Bedford, MA; diluted 1:7 with serum-free Ham’s F-12 nutrient mixture and Dulbecco’s modified Eagle’s medium (F-12/DMEM, 1.1, v/v), Life Technologies, Inc.-coated 12-well dishes at a density of 0.5 × 10⁶ cells/cm² in F-12/DMEM supplemented with gentamicin (20 μg/ml), sodium bicarbonate (1.2 g/ml), 15 μM HEPES, bovine insulin (10 μg/ml), human transferrin (5 μg/ml), batracin (5 μg/ml), and epidermal growth factor (2.5 ng/ml). Cells were incubated at 35 °C in a humidified atmosphere of 95% air and 5% CO₂. For low density Sertoli cell cultures where specialized tight junctions did not form but cell-cell and cell-substratum adhering and communicating junctions were present, isolated cells were plated in 100-mm dishes at a density of 5 × 10⁵ cells/cm² in F-12/DMEM supplemented with factors as described above. To obtain Sertoli cells with a purity of greater than 95%, cultures were hypotonically treated 48 h after plating with 20 mM Tris, pH 7.4, at 22 °C for 2.5 min to lyse contaminating germ cells (14). This was followed by two successive washes with F-12/DMEM. Media were replaced every 24 h thereafter. Day 0 of Sertoli cell cultures represents 24 h after the hypotonic treatment. These high and low cell density cultures were then incubated for 6–10 liters of SCCM routinely obtained from about 40–60 rats were collected on days 4 and 8 were stored at −80 °C until use in testicular cell cultures.

**Preparation of Sertoli Cell-conditioned Medium (SCCM)—**SCCM was prepared as described previously (15) using primary Sertoli cell cultures from 20-day-old Harlan Sprague-Dawley rats. The resulting Sertoli cells were plated in 100-mm dishes at a density of 5 × 10⁴ cells/cm². Cultures were hypotonically treated 48 h after plating. Therefore, Sertoli cells were cultured for an additional 8 days. Median collected on days 4 and 8 were stored at −20 °C until use. Batches of 6–10 liters of SCCM routinely obtained from about 40–60 rats were pooled, concentrated, and equilibrated against 20 mM Tris, pH 7.4, at 22 °C as described (13) for subsequent immunoblot analysis.

**Preparation of Cytosols and Membrane Extracts—**Cytosols were prepared by primary cultures of Sertoli cells, germ cells, and adult rat testes. Briefly, cells and tissue were suspended in ice-cold 10 mM Tris containing 1 mM EDTA, 5 mM phenylmethylsulfonyl fluoride (a serine protease inhibitor), and 1 mM PMSF, pH 7.6. The suspension was homogenized with 3–5 strokes in a glass homogenizer and a Teflon-coated pestle. Samples were then centrifuged at 45,000 × g for 30 min to remove membrane debris and filtered through a 0.2-μm filter unit. Membrane extracts were obtained by solubilizing pellets obtained in the above step in a lysis buffer (0.125 M Tris, pH 6.8, at 22 °C containing 1% SDS (w/v)). Protein estimation was performed by the Coomassie Blue dye binding assay (24) using bovine serum albumin as a standard.

**mRNA Differential Display and Cloning of Sertolin cDNA**

mRNA differential display was performed essentially as described (8–10) using RNAs isolated from primary cultures of Sertoli cells, germ cells, and Sertoli-germ cell cocultures. Sertoli and germ cells were prepared as described above using rats at 20 and 27 days of age, respectively. Cocultures of Sertoli-germ cells were prepared by layering germ cells onto Sertoli cells (which had previously been incubated for 5 days to allow the formation of specialized inter-Sertoli junctions) for 2 days before RNA was extracted for use in mRNA differential display experiments. Briefly, RNAs were reverse transcribed into cDNAs utilizing an oligo(dT₃₃)CA primer. Subsequent PCR was performed using a random sense primer of 5′-CTTCGCGATCCTG-3′ (nucleotides 56–67; see Fig. 2A) and oligo(dT₃₃)CA with 5′-[35S]ATP (specific activity, 1370 Ci/mmol; Amersham Pharmacia Biotech) to amplify cDNAs. The cycling parameters for the PCR were as follows: denaturation at 94 °C for 1 min, annealing at 42 °C for 2 min, and extension at 72 °C for 3 min. A total of 40 cycles were performed. The cycles were followed by a 15-min extension period at 72 °C. Aliquots of resultant PCR products were resolved by PAGE on a 8%–12% polyacrylamide gel. PCR products were visualized by autoradiography. A cDNA clone of 676 bp, designated sertolin, which was detected in Sertoli cells and Sertoli-germ cell cocultures but apparently present at a low abundance in germ cells, was selected, electroeluted from the gel, reamplified by PCR, and subcloned into pGEM-T™ vector (Promega, Madison, WI) for nucleotide sequencing. Nucleotide sequencing was performed by the dyeoxynucleotide chain termination method using Sequenase™.
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(Amersham Pharmacia Biotech) as described previously (25). This 676-bp clone contained about 90% of the entire open reading frame of sertolium mRNA. Differential display experiments using RNAs isolated from different batches of Sertoli, germ, and Sertoli-germ cell cultures were repeated five times, and the band corresponding to sertolium was seen in three of these experiments. To confirm that the cDNA was not a mere artifact, another sertolium cDNA was isolated using the Marathon kit (CLONTECH, Palo Alto, CA) according to the instructions provided by the manufacturer using a sertolium antisense primer of 5′-ATTTACATCA-CAGTTATCCACTGTA-3′ (nucleotides 215–239), which isolated a clone of about 1.4 kilobase. Briefly, 1 μg of Sertoli cell poly(A)+ RNA isolated by the mRNA Separator kit (CLONTECH) was used for double-stranded cDNA synthesis. Subsequent PCR was performed using the Advantage cDNA polymerase Mix (CLONTECH). In addition to the two sertolium cDNA clones obtained and described above for sequencing, we have subsequently isolated a third sertolium cDNA by PCR containing the entire open reading frame to confirm the sertolium nucleotide sequence.

Preparation of Sertolium Synthetic Peptide and Polyclonal Antibody Production

To confirm that the isolated sertolium cDNA indeed translates into a protein found in Sertoli cells and/or germ cells corresponding to the calculated M₀ of sertolium based on its deduced amino acid sequence, a 22-amino acid internal peptide of NH₂-KKEHFNLFKAASVSHLVQV-VPQ based on the primary sequence for sertolium was synthesized (SynPep Corp., Dublin, CA), which shared no significant homologies when compared with the existing peptide sequences in data bases at BLAST and Protein Identification Resource (PIR). To purify the synthetic peptide, about 300 μg of the crude peptide was loaded onto a Vydac™ (Separations Group, Hesperia, CA) C18 reverse-phase HPLC column (4.6 × 250-mm inner diameter) at a flow rate of 1 ml/min. The bound peptide was eluted using a linear gradient of 30–70% solvent B (95% acetonitrile, 5% water containing 0.1% trifluoroacetic acid, v/v) for a period of 30 min. The eluents were monitored by UV absorbance at 220 nm, and fractions of 0.5 ml were collected. Thereafter, about 0.1 nmol of the purified peptide was sequenced as described previously (13, 26, 27) using an Applied Biosystems 473A pulsed-liquid phase sequencer (Applied Biosystems, Foster City, CA) to confirm its sequence. Phenylhydantoin-derivatives were identified and quantified by HPLC utilizing a Brownlee™ PTH-C18 (2.1 × 220-mm inner diameter) column (Perkin-Elmer/Applied Biosystems). Protein sequencing was repeated twice using two different batches of the purified sertolium synthetic peptide. The repetitive yield was about 96%. Thereafter, a total of about 2 mg of this purified peptide was conjugated to carboxymethyl bovine serum albumin at a peptide-carrier ratio of 1:1 via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDC) using the Imject SuperCarrier™ EDC System for Peptides (Pierce). Subsequently, the peptide-carrier conjugate was purified by gel filtration and used for immunization (28) in a New Zealand White female rabbit. Preliminary detection of specific antigen-antibody interactions was determined by immunodiffusion assay as detailed elsewhere (29). This antibody was used for immunoblotting to see if it reacted with a protein that was similar to the calculated M₀ of sertolium based on its deduced amino acid sequence as described elsewhere (30).

Treatment of High Density Sertoli Cell Cultures with Cytokines

We proceeded to examine the effects of recombinant IL-1α (specific activity, 1 × 10⁸ units/mg of protein), INF-γ (specific activity, 1 × 10⁹ units/mg of protein), and bFGF (Calbiochem, La Jolla, CA) on Sertoli cell sertolium expression in vitro, since germ cells are known to synthesize and/or express multiple cytokines that can affect testicular function (31–33). Sertoli cells, prepared essentially as described above, were cultured on Matrigel™-coated 12-well dishes at a density of 0.5 × 10⁶ cells/cm². Cultures were hypotonically treated 48 h thereafter. Sertoli cells were then incubated for an additional 24 h to allow for cell recovery. Thereafter, Sertoli cells were cultured for an additional 0–24 h in the presence of IL-1α (10 units/dish), INF-γ (100 units/dish), or bFGF (150 ng/dish). Total RNA was subsequently extracted from these cells for RT-PCR. Control experiments included Sertoli cells cultured alone in duplicate wells under the same conditions as described above without the addition of any factors or with the addition of vehicle (ethanol) only.

Induction of Generalized Inflammation in Adult Rats by Injecting Fermented Yeast

Experimental inflammation was induced in adult male Harlan Sprague-Dawley rats (250-300 g, body weight) by injection with fermented yeast as described previously (34). Briefly, rats were injected subcutaneously at multiple sites with 10 ml/kg body weight of 10% (gm/ml) fermented brewer’s yeast suspended in sterile water. Fermentation was allowed to proceed at 37 °C for approximately 15 h. Thereafter, the fermented yeast was stored at 4 °C for at least 24 h before its use. Animals were killed by CO₂ asphyxiation, and testes, brain, and liver were removed at 2, 6, 24, 48, and 96 h after the injection of yeast. Tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use for RNA extraction.

RT-PCR

Total RNA was extracted from cells and tissues using RNA STAT-60™ as described previously (15). RT-PCR was performed essentially as described previously (5, 13). Briefly, 2 μg of total RNA was reverse transcribed into cDNAs using 5 μg of oligo(dT)₁₂, and a Moloney murine leukemia virus reverse transcriptase kit (Promega) in a final reaction volume of 25 μl. From this reaction product, 3 μl was used and served as a template for PCR in combination with 0.3 μg each of the sertolium sense and antisense primer pair coamplified with the rat ribosomal S16 primer pair. The primers used for the amplification of sertolium and S16 (32, 33) were as follows: 5′-5AATCTTGAGGTCCTGCTGAA-3′ (sertolium, sense, nucleotides 56–85), 5′-GAGTTACGGCGTTCGAA-3′ (sertolium, antisense, nucleotides 254–271), 5′-TCCGCTGACCTCGTTAACCTGTCCT-3′ (S16, sense, nucleotides 15–38), and 5′-GGCAACATCTCTGGATTGCAGCAGC-3′ (S16, antisense, nucleotides 376–399). Coamplification with S16 was included to ensure that equal amounts of RNA were reverse transcribed and amplified in each reaction tube. The cycling parameters for the PCR reaction were as follows: denaturation at 94 °C for 1 min, annealing at 61 °C for 2 min, and extension at 72 °C for 3 min. A total of 25 cycles were performed. The cycles were followed by an extension period at 72 °C for 15 min. Aliquots of 5–10 μl were resolved onto 5% T polyacrylamide gels in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0, at 22 °C). In some instances, an aliquot from the RT product was used as a template for hot nested PCR as described previously (5, 13, 36). About 0.2 μg of the antisense primer was 5′-end-labeled with [γ-³²P]ATP (specific activity, 6000 Ci/mmol; Amersham Pharmacia Biotech) by utilizing T4 polynucleotide kinase (Promega). Antisense S16 was also 5′-end-labeled for coamplification as described above. Under these conditions, the amplifications of sertolium and S16 were both in the linear range, as verified in preliminary experiments when an aliquot of 10 μl of PCR product was withdrawn from each of the PCR tubes in cycles 20, 22, 25, 27, and 30 for gel analysis. PCR products were visualized by either ethidium bromide staining or autoradiography using X-OMAT® AR™ X-ray film (Eastman Kodak Co.).

Northern Blot Analysis

Total RNA was extracted from Sertoli cells (isolated from 20-day-old rats) and germ cells (isolated from adult rat testes) and cultured from rats of different ages using RNA STAT-60™ as described previously (15). A total of 20 μg of RNA was used for analysis. A partial Sertoli cell sertolium cDNA of 216 bp was prepared by RT-PCR using a primer pair for sertolium (see Fig. 2A), was nick-translated, and was used for hybridization. This 216-bp cDNA was previously confirmed to be sertolium cDNA when it was electrophoresed and blotted onto nylon membranes. Autoradiography revealed that 0.5–1 × 10⁶ cpm/ml of the ³²P-labeled probe was used for hybridization overnight at 42 °C. The blots were then washed twice in double-strength SSC (0.3 M sodium chloride, 30 mM sodium citrate, pH 7.0, at 22 °C) at room temperature for 5 min, twice in double-strength SSC containing 1% SDS at 60 °C for 40 min, and twice in 0.1× SSC at room temperature for 30 min. The blots were then exposed to x-ray film at −80 °C.
General Methods

Analytical PAGE in the presence of SDS was performed as described previously (37, 38). The resolving gel consisted of 12.5% T (total acrylamide concentration) and 2.6% cross-linker using N,N'-methylene-bis-acrylamide with a stacking gel of 5% T and 15% N,N-diallyltartardiamide (Bio-Rad). All aliquots, unless otherwise noted, were denatured and reduced in SDS sample buffer (0.125 M Tris, pH 6.8, at 22 °C containing 1% SDS (w/v), 1.6% 2-mercaptoethanol (v/v) and 10% glycerol (v/v)). Polyacrylamide gels were silver-stained as detailed elsewhere (39). Hydropathy analysis was performed using DNASIS (version 7.0, Hitachi Software Engineering America, Ltd., San Francisco, CA) according to Kyte and Doolittle (40). Densitometric scannings of autoradiograms were performed using an UltroScan XL Enhanced Laser Densitometer (Pharmacia Amersham Biotech) at 600 nm. Statistical analysis was performed by Student's t test using the GB statistical analysis software package (version 3.0, Dynamic Microsystems, Inc., Silver Spring, MD).

RESULTS

Cloning, Nucleotide Sequence Analysis, and Partial Characterization of Rat Testicular Sertolin—Utilizing mRNA differential display and PCR, we were able to identify and clone a novel cDNA designated sertolin when using RNAs isolated from primary Sertoli cells, germ cells, and Sertoli-germ cell cocultures (Fig. 1). This cDNA as visualized in the autoradiogram (Fig. 1, arrow) was detected in Sertoli cells and Sertoli-germ cell cocultures with apparently low expression in germ cells. The full-length cDNA of 853 bp containing 122-bp 5′-untranslated and 350-bp 3′-untranslated regions with an open reading frame of 381 bp coded for a 127-amino acid polypeptide of 13,759 daltons with an estimated pl of 6.374 (Fig. 2A). Sequence analysis of several sertolin clones revealed the existence of another molecular variant. Amino acid residues at Asp52 (GAT), Ala110 (GCC), and Val127 (GTT) found in two clones were determined as follows: Gly52 (GGT), Thr110 (ACC), and Ile127 (ATT) in another clone of 570 bp. Comparison of the full-length cDNA and deduced amino acid sequences with the existing data bases at BLAST, GenBankTM, and PIR showed a 20% amino acid homology in a short stretch of sequence between amino acids 88 and 121 with the rat spinocerebellar ataxia type 3 antitoxin protein (amino acid residues 30–63) and its human homolog, the Machado-Joseph Disease josphin protein (amino acid residues 29–62) known to be involved in neurodegeneration (Fig. 2B). In addition, another short stretch of sertolin cDNA (amino acid residues 39–85) shared a 40% homology with human thrombospondin (amino acid residues 232–282), an extracellular matrix glycoprotein (Fig. 2B). Hydropathy analysis according to Kyte and Doolittle revealed that the overall sertolin amino acid sequence is hydrophobic with about 16 hydrophobic domains and a mean hydropathic index of 0.17 (Fig. 2C). To confirm that the sertolin cDNA can indeed translate into a protein of about 14 kDa (calculated M, 13,759), we prepared a 22-amino acid internal synthetic peptide of NH2-KKEHPNFLFKAASVSHLQVQVPQ, which shared no significant homologies with existing proteins in the data base at GenBankTM, for the production of a polyclonal antibody. This synthetic peptide was purified to apparent homogeneity by a reverse-phase HPLC column and eluted in fractions 19 and 20 under protein peak 4 (Fig. 3A, solid bar). Partial NH2-terminal amino acid sequence analysis of this peptide yielded a sequence of NH2-LKEFPNFLKAXSV (Fig. 3A), indicating that the peptide that eluted from the column under peak 4 is indeed the sertolin peptide that was synthesized. Thereafter, the purified 22-amino acid sertolin peptide was conjugated to cationized bovine serum albumin for subsequent immunization in a rabbit. When aliquots of proteins derived from Sertoli and germ cell-conditioned media and Sertoli cell and testicular cytosols were resolved by SDS-PAGE under reducing conditions and silver-stained, an extremely complex pattern of proteins was noted in these samples (Fig. 3B). Moreover, the monospecificity of the anti-rat sertolin antibody was confirmed when these samples were electrophoretically transferred onto a nitrocellulose membrane for immunoblot analysis. A single 17-kDa immunoreactive protein similar to the calculated M, of sertolin (13,759 daltons) based on its deduced amino acid sequence (Fig. 2A) was only detected in the Sertoli cell cytosol (Fig. 3C) but not in germ cell cytosol or Sertoli cell membrane extract (data not shown). Sertolin was also not found in the Sertoli cell-conditioned medium, suggesting that it may not be a secretory...
Fig. 2. Characterization of the sertolin cDNA. A, nucleotide sequence of the 853-bp sertolin cDNA containing 122-bp 5'- and 350-bp 3'-untranslated regions with an open reading frame of 381 bp coding for a 127-amino acid polypeptide. The boldface, double-underlined sequence (nucleotides 56–67) is the random sense primer that was used in conjunction with the oligo(dT)$_{12}$CA for mRNA differential display and PCR to identify the sertolin cDNA. The double-underlined sequences (nucleotides 56–85 and 254–271) are the primer pair used for all the RT-PCR studies. The boxed sequence (amino acid residues 100–121) displays the amino acid sequence of the partial, internal synthetic peptide that was prepared for subsequent sertolin antibody production. The underlined sequence (nucleotides 215–239) is the antisense primer that was used to isolate...
Moreover, the immunoreactive detection of this 17-kDa protein was only possible if the Sertoli cell cytosol was concentrated by severalfold, suggesting that the abundance of sertolin in the Sertoli cell cytosol is extremely low, which may explain its absence in the testicular cytosol (Fig. 3C). As such, these data are in agreement with the semiquantitative RT-PCR results shown in Fig. 6, A and B (see below) examining the sertolin mRNA distribution in various tissues and testicular cells. Furthermore, subunit structural analysis under reducing and nonreducing conditions by immunoblot analysis showed the presence of a single immunoreactive band of 17 kDa in the Sertoli cell cytosol, indicating that sertolin consists of a single polypeptide chain (data not shown).

Developmental Regulation of Sertolin in the Testis—Since in the developing testis there is a drastic increase in Sertoli-germ cell interactions at the onset of spermatogenesis, we examined another sertolin cDNA clone by the Marathon kit to confirm the sertolin sequence as described under "Experimental Procedures." Amino acid residues 52 (Asp), 110 (Ala), and 127 (Val) as denoted by shaded boxes indicate the existence of another sertolin molecular variant. B, comparison of short stretches of amino acid sequence (amino acid residues 88–121 and 39–85) of sertolin with rat spinocerebellar ataxia type 3 antaxin protein (amino acid residues 30–63), human Machado-Joseph Disease josephin protein (amino acid residues 29–62), and human thrombospondin protein (amino acid residues 232–282). Sertolin shared 20% amino acid homologies with rat antaxin protein and its human homolog, josephin protein. In addition, sertolin also shared a 40% homology with human thrombospondin, an extracellular matrix protein. C, hydropathy plot of sertolin. Hydropathic analysis revealed that sertolin contains 16 hydrophobic domains with a mean hydropathic index of 0.17 as indicated by the horizontal line.
the steady-state sertolin mRNA level in the developing testis from 1 to 90 days of age to assess if sertolin expression correlates with the onset of spermatogenesis during maturation. Northern blot analysis revealed the presence of two sertolin mRNA transcripts of 2.3 and 1 kilobase in Sertoli cells and germ cells isolated from adult rat testes (Fig. 4, A and B). These studies also illustrated an increase in both mRNA transcripts during testicular maturation which peaked at 20–90 days of age (Fig. 4, C and D). However, the expression of sertolin was virtually undetectable in 1–10-day-old rat testes (Fig. 4, C and D). When these studies were repeated by semiquantitative RT-PCR and the relative steady-state mRNA level of sertolin in the testis was corrected to take into account age-related increases in testicular weight, the steady-state sertolin mRNA level per pair of testes increased by as much as 8–15-fold from 45 to 90 days of age when compared with 20-day-old rats (Fig. 4E), illustrating that the increase in testicular sertolin expression coincides with the onset of spermatogenesis. This was done in order to take into account the drastic increase in the germ cell:Sertoli cell ratio number at the onset of spermatogenesis at 45 days of age, which results in a 7.5-fold increase in testicular weight (pair of testes) when compared with 20-day-old rats.

Expression of Sertolin in Sertoli Cells Cultured at High and Low Cell Density—Previous reports have shown that Sertoli cells cultured at high cell density (0.5 × 10^6 cells/cm^2) on Matrigel™-coated bicameral units mimic their morphological and physiological characteristics in vivo (41, 42). Moreover, when Sertoli cells were cultured at high cell density, there were striking changes in the expression of several junctional complex component genes such as zonula occludens-1 (ZO-1), N-cadherin, and connexin-33 at the time of specialized junction formation (44). We therefore examined the steady-state sertolin mRNA level during the formation of specialized junctions such as occluding, anchoring, and communicating junctions between Sertoli cells to assess whether sertolin is a possible marker to probe these events. We failed to detect any changes in the steady-state sertolin mRNA level when Sertoli cells were cultured alone at high cell density (0.5 × 10^6 cells/cm^2), illustrating that sertolin is probably not a marker to monitor the events involved in specialized junction formation between Sertoli cells (data not shown). Similar results were obtained when Sertoli cells were cultured at low cell density (5 × 10^4 cells/cm^2) (data not shown) where occluding tight junctions did not form but adhering and communicating gap junctions were present.

Expression of Sertolin in Sertoli Cells Cocultured with Germ Cells—Because there is an increase in the steady-state sertolin mRNA level in the developing testis, we examined whether the increase in the sertolin mRNA level is due to an up-regulation by germ cells. Previous studies from this laboratory have shown that when germ cells are co-cultured with Sertoli cells for short periods of time up to 24 h prior to the establishment of Sertoli-germ cell junctions, there were surprising and significant changes in the mRNA expression of several genes such as cathepsin L (5), a cysteine protease that has been implicated in several pathological conditions such as tumor growth and metastasis (45). Therefore, we considered it important to examine whether short term interactions between Sertoli and germ cells would influence the steady-state sertolin mRNA level. Sertoli cells cultured at high cell density were first allowed to form intercellular junctions for 4 days in vitro before adding germ cells freshly isolated from 27-day-old rats at a ratio of 1:1 for periods of up to 24 h. Time 0 of Sertoli-germ cell co-cultures represents dishes where RNA STAT-60™ was added to Sertoli cells immediately following the addition of germ cells at a Sertoli:germ cell ratio of 1:1 (contribution of RNA from germ cells is 50% at time 0). Cocultures were termi-

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C. C. S. Wong, S. S. W., Chung, L-J. Zhu, W. M. Lee, and C. Y. Cheng, submitted for publication.
FIG. 5, Changes in the steady-state sertolin mRNA level when germ cells are cocultured with Sertoli cells (SC-GC). Sertoli cells were cultured at 0.5 × 10⁶ cells/cm² on Matrigel™-coated dishes as described under “Experimental Procedures.” Cells were cultured for an additional 5 days to allow the establishment of specialized inter-Sertoli junctions prior to their use in coculture experiments. Freshly isolated germ cells from 27-day-old rats were cocultured with Sertoli cells for 0.5, 1, 2, 3, 4, 5, 6, and 24 h. A, an autoradiogram of RT-PCR demonstrating that a 3–5-fold decrease in sertolin expression was noted only at 3–4 h after the addition of germ cells to the Sertoli cell culture at the time of germ cell attachment to Sertoli cells. Co-amplification was performed using a S16 primer pair. B, densitometric scanings of three different autoradiograms of RT-PCR such as the one shown in A normalized against S16. C, to confirm that any changes in sertolin expression detected in Sertoli-germ cell cocultures as seen in A and B were not due to an endogenous change in sertolin expression in Sertoli cell cultures, Sertoli cells were cultured under the same conditions as described above but without the addition of any germ cells. Densitometric scanings of at least three different autoradiograms of RT-PCR were normalized against S16 from this set of experiments, demonstrating that Sertoli cells (0.5 × 10⁶ cells/cm²) cultured in the absence of germ cells did not exhibit any significant changes in sertolin expression. *, significantly different from control SC-GC at 0 h in A and B or SC alone at 0 h in C. p < 0.01; ns, not significantly different from control.
nated, while germ cells were allowed to attach to Sertoli cells prior to the formation of specialized junctions. It was demonstrated that there was a 3–5-fold reduction in the steady-state sertolin mRNA level only at 3–4 h after the addition of germ cells at the time of germ cell attachment (Fig. 5, A and B). Experiments using fluorescein-labeled germ cells have demonstrated that germ cells begin to bind to Sertoli cells at 1–3 h (21). Nonetheless, the expression of sertolin in our Sertoli-germ cell cocultures returned to its basal level by 5 h and remained at that level thereafter. Sertoli cells cultured alone in the absence of germ cells failed to elicit any changes in the steady-state sertolin mRNA level (Fig. 5C). As such, these changes are likely to be mediated by germ cells. However, the incubation of increasing concentrations of GCCM at concentrations of 20, 100, and 400 μg of total protein/dish for 4 and 24 h (data not shown) failed to decrease the expression of sertolin suggesting the changes detected in the coculture experiments described above are probably mediated via cell-cell contact.

**Cellular and Tissue Distribution of Sertolin**—The cellular and tissue distribution of sertolin mRNA in various cells and tissues from adult male and female rats was examined by RT-PCR (Fig. 6, A and B). A 216-bp PCR product corresponding to the expected size of sertolin mRNA was found in the lung, adrenal gland, ovary, testis, Sertoli cells, germ cells, brain, kidney, spleen, liver, and epididymis. Sertolin expression was either not detected or barely detected in the uterus, heart, and thoracic duct. It is worthy to note that the highest expression of sertolin was detected in the Sertoli cell (Fig. 6, A and B).

**Regulation of Sertoli Cell Sertolin Expression by Hormones and Sex Steroids in Vitro**—Interestingly, a survey of several tissues and cells by RT-PCR was able to reveal that the steady-state mRNA level of sertolin was slightly higher in the female such as in the brain, kidney, and spleen (Fig. 6, A and B) than in the male counterpart. Likewise, it should also be noted that the expression of sertolin in the male rat was the highest in the Sertoli cell (Fig. 6, A and B). Therefore, we assessed the effects of various sex steroids, in addition to FSH, on the steady-state sertolin mRNA level in Sertoli cells (Fig. 7, A–D) to determine whether these differences in sertolin expression in the male and female are due to androgens, estrogens, and progestins. FSH at 100 ng/dish (data not shown) and 17β-hydroxy-5α-androstan-3-one at 1 x 10^{-7} M (data not shown) were both unable to significantly affect sertolin mRNA expression when incubated with Sertoli cells for up to 24 h. However, both estradiol-17β (Fig. 7, A and B) and progesterone (Fig. 7, C and D) at 1 x 10^{-7} M when incubated with Sertoli cells were able to evoke a mild but statistically significant stimulation in sertolin expression at 5–24 h and 2–24 h, respectively, suggesting that this gene may be regulated by female sex steroids in vivo. These changes are probably not due to an artifact, since Sertoli cells...
cultured alone under the same conditions without the addition of any sex steroids exhibited no changes in the steady-state sertolin mRNA level (data not shown).

**Regulation of Sertoli Cell Sertolin Expression by IL-1α, INF-γ, and bFGF in Vitro**—Several reports have implicated cytokines synthesized and/or expressed by Sertoli and/or germ cells to be known regulators of testicular function (31–33). For instance, preliminary experiments have demonstrated that cytokines are capable of influencing several proteases and protease inhibitors, among them bFGF. It was found that the steady-state sertolin mRNA level in rat Sertoli cells cultured in vitro was unresponsive to either INF-γ (100 units/dish) or bFGF (50 ng/dish) (data not shown). However, IL-1α (10 units/dish) induced a transient but significant increase in the Sertoli cell sertolin steady-state mRNA level (Fig. 8, A and B). By 2 h, the level of sertolin expression increased almost 3-fold that of the control, which returned to the basal level by 4 h (Fig. 8, A and B) and remained at that level thereafter. These changes probably are not due to an artifact, since Sertoli cells cultured under the same conditions alone without the addition of any cytokines exhibited no changes in the steady-state sertolin mRNA level (data not shown).

**Regulation of Sertolin during Experimentally Induced Generalized Inflammation**—Since there was a transient but significant increase in the steady-state sertolin mRNA level when Sertoli cells were treated with IL-1α (10 units/dish) (Fig. 8, A and B) and previous reports have implicated cytokines to be involved in the inflammatory response (46–50), we proceeded to examine sertolin mRNA expression in the testis (Fig. 9, A and B), brain (data not shown), and liver (data not shown) when we induced general inflammation in the adult rat by the injection of fermented yeast. In the testis (Fig. 9, A and B), there was a 4–14-fold increase in the sertolin steady-state mRNA level from 2 to 48 h; however, the steady-state sertolin mRNA level in the brain (data not shown) and liver (data not shown) were both shown to be unresponsive to the inflammatory response. In any case, these results illustrate that the response of the testis to induced inflammation is unique, and sertolin may possibly be an acute phase marker in the testis.

**DISCUSSION**

In the present study, we report the cDNA cloning of a novel Sertoli cell gene product designated sertolin. Comparison of the nucleotide and translated amino acid sequences with the existing data bases at BLAST, GenBank™, and PIR revealed that sertolin shared about a 20% homology at the amino acid level in a short stretch of sequence (amino acid residues 88–121) with the rat spinocerebellar ataxia type 3 antaxin protein (amino acid residues 30–63) (52, 53) and its human homolog, the Machado-Joseph disease josephin protein (amino acid residues 29–62) (54–57), a predominantly cytoplasmic protein that is widely distributed in human neurons but also detected in the nuclei of neurons and glial cells (58, 59). Machado-Joseph disease, in addition to Huntington’s disease (60), are two of several neurodegenerative diseases where expanded CAG repeats are found to associate with these disorders that translate into polyglutamine stretches (61–63). Although it is speculated that the CAG repeats are responsible for these and numerous other neurodegenerative diseases, the exact mechanism(s) by which they induce these disorders remains largely unelucidated. Interestingly, others were able to detect mRNA corresponding to the Machado-Joseph disease gene in nonneuronal tissues such as the heart, liver, kidney, spleen, and testis (64) from normal

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3 D. D. Mruk and C. Y. Cheng, unpublished observations.
4 I. Schmitt, T. Brattig, M. Gossen, and O. Reiss, GenBank™ accession number Y12319.
individuals, suggesting that these genes may indeed have multiple biological actions. Recently, phosphoneuroprotein 14, a 14-kDa protein abundant in the brain (65, 66), was also found to be present in cultured rat Sertoli cells having a fibrillar pattern in the cytoplasm and around the nuclei, which did not seem to resemble actin stress fibers, microtubules, or intermediate filaments, and the rat testicular phosphoneuroprotein 14 was up-regulated between the day of birth and 4 weeks of age (67). Likewise, Beer et al. (68) reported the immunohistochemical localization of yet another neurodegenerative protein that is involved in Alzheimer's disease known as amyloid precursor protein in Sertoli cells, follicle cells, and macrophages. Because of the ubiquitous expression of amyloid precursor protein in several different cell types, it was speculated that amyloid precursor protein may be involved in tissue maintenance and repair (68).

In addition to spinocerebellar ataxia type 3 antaxin protein and Machado-Joseph disease joesphin protein, sertolin also shared a 40% homology in a short stretch of sequence (residues 39–85) with human thrombospondin (residues 232–282), an extracellular matrix protein, known to be involved in cell adhesion, proliferation, and migration (for reviews, see Refs. 69 and 70). Thrombospondin is present in high concentrations in platelet α-granules but is also known to be synthesized by other cell types in vitro (71). In addition, studies have demonstrated that the synthesis of thrombospondin by adherent cells in culture is highly regulated and appears to be cell cycle-dependent (72). Recent evidence suggests that thrombospondin may serve as a matrix or cell-bound template for the activation of plasminogen by tissue plasminogen activator (73, 74), a serine protease known to be synthesized and secreted by Sertoli cells (75). To date, however, there appear to be no reports localizing thrombospondin mRNA or protein to the testis.

We previously reported that short term interactions of Sertoli and germ cells in vitro prior to the formation of specialized junctions such as desmosome-like (76) and gap junctions (77), which are known to form between 24 and 48 h in vitro (20, 21), are associated with transient but significant changes in the

![FIG. 8. Regulation of the sertolin steady-state mRNA level in Sertoli cells (SC) by IL-1α (10 units/dish) in vitro. A, an autoradiogram of a RT-PCR showing a transient but significant increase in sertolin mRNA expression at 2–3 h after the addition of IL-1α, whereas the addition of INF-γ (100 units/dish) and bFGF (150 ng/dish) elicited no significant changes on Sertoli cell sertolin expression (data not shown). Co-amplification was performed using a S16 primer pair. B, densitometric scanning of at least three different autoradiograms of RT-PCR such as the one shown in A normalized against S16. *, significantly different from control at 0 h without the addition of IL-1α; p < 0.001; ns, not significantly different from control at 0 h without the addition of IL-1α.](image)

![FIG. 9. Changes in the steady-state sertolin mRNA level in the testis when rats were induced with inflammation by injection with fermented yeast. Induction of generalized inflammation was performed as described under “Experimental Procedures.” A, an autoradiogram of RT-PCR showing that there was a striking increase in the steady-state mRNA level of sertolin in the adult testis from 2 to 96 h illustrating that sertolin is an acute phase protein in the testis. Co-amplification was performed using a S16 primer pair. B, densitometric scannings of at least three different autoradiograms of RT-PCR such as the one shown in A normalized against S16. *, significantly different from control at 0 h without experimentally induced inflammation, p < 0.001.](image)
expression of several proteases and protease inhibitors (5). Using Sertoli-germ cell cocultures, it was shown that there were transient but significant increases in both serine and cysteine protease activities as well as in the expression of selected proteases and protease inhibitors at 3–6 h (5) coinciding with the time when germ cells attach to Sertoli cells, but prior to the establishment of specialized junctions, illustrating that the formation of intercellular junctions is a dynamic event that requires the participation of multiple biological factors (5). This conclusion was reached based on the fact that Sertoli cells cultured alone under these conditions failed to display any changes in the expression of these proteases or protease inhibitors (5). Because the movement of developing germ cells from the basal to the adluminal compartment in the seminiferous epithelium consists of intermittent phases of junction disassembly and reassembly, these studies clearly illustrate the potential that a disruption of a selected set of genes can disrupt the timely movement of germ cells across the epithelium.

Herein we report that when germ cells were cocultured with Sertoli cells, the expression of sertolin was found to decline by as much as 5-fold only at 3–4 h, at the time when the expression of selected proteases such as urokinase-plasminogen activator, cathepsin L, and trypsin was high (5). The fact that Sertoli cells cultured alone failed to display any changes in sertolin expression seemingly suggests that germ cells are capable of modulating sertolin expression either directly or indirectly. In addition, studies presented herein demonstrate that this transient decrease in sertolin expression at 3–4 h is not likely to be mediated via a germ cell soluble factor(s) since the incubation of increasing concentrations of crude GCCM at 20–400 μg/ml total protein/dish failed to elicit a change in the sertolin steady-state mRNA level (data not shown). This latter result further supports the postulate that the transient but drastic decline in sertolin expression in the short term cocultures is mediated by cell-cell contact and may possibly be related to the formation of specialized Sertoli-germ cell junctions. As such, sertolin is likely an important candidate molecule to monitor the events of germ cell movement.

While these observations seemingly suggest the involvement of proteases and protease inhibitors in junction formation, proof of their direct involvement is lacking. However, recent studies from this laboratory have shown that the inclusion of either α2-macroglobulin (a nonspecific protease inhibitor and also a Sertoli cell product) (78, 79) or aprotinin (a serine protease inhibitor, cathepsin L, and trypsin was high (5). The fact that Sertoli cells cultured alone failed to display any changes in sertolin expression seemingly suggests that germ cells are capable of modulating sertolin expression either directly or indirectly. In addition, studies presented herein demonstrate that this transient decrease in sertolin expression at 3–4 h is not likely to be mediated via a germ cell soluble factor(s) since the incubation of increasing concentrations of crude GCCM at 20–400 μg/ml total protein/dish failed to elicit a change in the sertolin steady-state mRNA level (data not shown). This latter result further supports the postulate that the transient but drastic decline in sertolin expression in the short term cocultures is mediated by cell-cell contact and may possibly be related to the formation of specialized Sertoli-germ cell junctions. As such, sertolin is likely an important candidate molecule to monitor the events of germ cell movement.

The expression of sertolin in developing testes may be due to an increase in Sertoli-germ cell interactions, since there is a drastic increase in the number of germ cells at the onset of spermatogenesis at 45–60 days of age. Interestingly, the two mRNA transcripts of 2.3 and 1 kilobase were barely detectable in 1–10-day-old testes. In addition, the increase in testicular sertolin expression at maturation may be also ascribed to Sertoli, Leydig, or peritubular myoid cells, a combination of these cell types, or possibly increased levels of expression within germ cells as they mature. Moreover, some other yet to be identified factor(s) may be responsible for the enhanced increase in sertolin expression during maturation. It remains to be determined whether Leydig and/or peritubular myoid cells express sertolin. Moreover, the immunoreactive detection of a 17-kDa protein corresponding to sertolin demonstrated that sertolin was a Sertoli cell cytosolic protein. These results, in combination with the molecular studies presented herein, demonstrate unequivocally the presence of such a molecule in the testis.

Since a survey of several tissues from both the female and male that included the brain, spleen, and liver illustrated that sertolin expression was slightly higher in the female counterpart, we assessed the effects of various sex steroids on Sertoli cell sertolin expression in vitro to determine whether these differences in sertolin expression in the male and female are due to regulation by sex steroids. In the rat testis, Leydig cells are known to be the major producers of androgens (80); however, it has also been demonstrated that Sertoli cells are capable of synthesizing and/or metabolizing several steroids such as estradiol (81), progesterone (82–84), and 17β-hydroxy-5α-androstan-3-one (85). Furthermore, FSH is a known regulator of testicular function (75), yet the addition of FSH (100 ng/dish) was unable to affect sertolin expression. In contrast, when Sertoli cells were cultured in the presence of estradiol-17β (1 × 10⁻⁷ M) and progesterone (1 × 10⁻⁷ M), there was a 2–2.5-fold increase in sertolin expression at 5–24 and 2–24 h, respectively. Based on these limited observations, it is difficult to speculate on the function of sertolin in the testis except that sertolin is modulated by both steroids and germ cell contact in addition to cytokines. Numerous studies have demonstrated that Sertoli- and germ cell-derived cytokines are capable of modulating testicular function. For instance, it has been reported that IL-1α, a known Sertoli cell and possibly a germ cell secretory product (86), was able to inhibit the secretion of both cyclic protein-2 (CP-2) and transferrin by Sertoli cells (87), whereas others have reported that IL-6, IL-2, and tumor necrosis factor were able to increase transferrin secretion by Sertoli cells (51). Cytokines have also been implicated in the inflammatory process (46–50). Data reported herein illustrate that neither INF-γ nor bFGF, both of which are germ cell-
derived cytokines, were able to influence Sertoli cell sertolin expression in vitro, since the addition of either one of these factors failed to affect sertolin expression in primary Sertoli cell cultures. However, the addition of IL-1α (10 units/dish) induced a transient but significant increase in sertolin expression at 2–3 h. This transient but significant increase in Sertoli cell sertolin expression is probably not due to an artifact, since Sertoli cells cultured under the same conditions without IL-1α failed to show any changes in sertolin expression.

Data presented herein demonstrated that sertolin expression increased from 4- to 14-fold from 2 to 48 h in the adult male rat after the induction of generalized inflammation in vivo. This is in sharp contrast to α2-macroglobulin, whose protein concentration in the serum and its expression increased drastically in response to inflammation in the brain and liver but not in the testis (15, 43, 78, 79). Hence, sertolin may be an acute phase protein in the testis, but not in the brain and liver. As such, sertolin may be a unique molecule to study the inflammatory response and other pathological conditions in the testis.

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