Testin Secreted by Sertoli Cells Is Associated with the Cell Surface, and Its Expression Correlates with the Disruption of Sertoli-Germ Cell Junctions but Not the Inter-Sertoli Tight Junction*

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Testin is a testosterone-responsive Sertoli cell secretory product. In the present study, we demonstrated that the amount of testin secreted by Sertoli cells in vitro was comparable with several other Sertoli cell secretory products. However, virtually no testin was found in the luminal fluid and cytosols of the testis and epididymis when the intercellular junctions were not previously disrupted, suggesting that secreted testin may be reabsorbed by testicular cells in vivo. Studies using Sertoli cells with and without a cell surface cross-linker and radiiodination in conjunction with immunoprecipitation illustrated the presence of two polypeptides of 28 and 45 kDa, which constitute a binding protein complex that anchors testin onto the cell surface. The 28- and 45-kDa peptide appear to be residing on and inside the cell surface, respectively. Immunogold EM studies illustrated testin was abundantly localized on the Sertoli cell side of the ectoplasmic specialization (a modified adherens junction) surrounding developing spermatids. In contrast, very few testin gold particles were found at the site of inter-Sertoli tight junctions. When the inter-Sertoli tight junctions were formed or disrupted, no significant change in testin expression was noted. This is in sharp contrast to the disruption of Sertoli-germ cell junctions, which is accompanied by a surge in testin expression. These results demonstrate the usefulness of testin in examining Sertoli-germ cell interactions.

Testin, a testosterone-responsive glycoprotein secreted by rat Sertoli cells in vitro, consists of two highly homologous variants with an apparent M\(_{r}\) of 35,000 and 37,000 (1–4). Immunofluorescent microscopy and immunohistochemistry reveal that testin resides near the basal lamina of the seminiferous epithelium in most stages of the cycle, consistent with its localization at the Sertoli-germ cell junction (5–6). However, a transient but drastic increase in testin accumulation was noted between Sertoli cells and the head of elongated spermatids at early stage VIII preceding spermiation (7), consistent with its localization at the ectoplasmic specialization, which is a modified adherens junction (see Table I). These observations suggest that testin may be a sensitive marker in examining the cellular events of Sertoli-germ cell interactions.

Once the mRNA sequence of testin was known, Northern blots and reverse transcription-polymerase chain reaction were used to survey the testin mRNA distribution in multiple organs from both adult and immature male and female rats. It was found that testin is predominantly expressed in the gonad (6). We postulated that this expression correlated with the rapid turnover of intercellular junctions in the testis and ovary during germinal cell development. This hypothesis was supported by the observations that testin mRNA can also be detected in non-gonadal tissues such as pre- and neo-natal rat kidney at the time of extensive tissue restructuring due to organ growth (8). Moreover, the steady-state testin mRNA level in the ovary is high at proestrus, estrus, and metestrus, correlating with the maturation of intrafollicular ova and the eventual rupturing of the follicle at ovulation that coincides with the rapid turnover of inter-granulosa cell junctions (8, 9). In addition, testin expression was drastically reduced to an almost undetectable level at diestrus, during which functional regression of the corpora lutea occurs (8, 9).

Other recent in vivo and in vitro studies reveal that testin is a sensitive marker to monitor the disruption of intercellular junctions in the testis, since the expression of testin is positively correlated to this event (8). For instance, a surge in testin expression and an intense accumulation of its protein in the cytosol of the testis are found when germ cells, mainly round and elongated spermatids, were depleted from the seminiferous epithelium by either lonidamine (8), busulfan (2, 3), or X-irradiation (10) at the time when Sertoli-germ cell junctions were disrupted. A brief hypotonic treatment lysing germ cells in Sertoli-germ cell cocultures, thereby disrupting the inter-Sertoli-germ cell junctions, also induced a drastic increase in testin expression by Sertoli cells (8). These studies, however, cannot distinguish whether the observed drastic increase in testin expression correlates with the disruption of inter-Sertoli tight junctions or adherens and gap junctions, which are found between Sertoli cells as well as between Sertoli and germ cells (Table I; for reviews, see Refs. 11–13). As such, we have used an established culture model that selectively disrupts tight junctions in vitro (14) by [Ca\(^{2+}\)] depletion using primary Sertoli cells cultured in vitro to assess whether such a disruption is associated with any changes in testin expression. In addition, we seek to (i) identify the distribution of testin in the epithelium by immunogold EM to define its subcellular localization, (ii) examine the effect of anti-testin IgG on the re-establishment of inter-Sertoli tight junctions after their disruption as assessed by the transepithelial electrical resistance (TER)
Testin Is a Sertoli-Germ Cell Junction Marker

MATERIALS AND METHODS

Animals and Preparation of Biological Fluids

Adult or immature Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Preparation of testicular and epididymal cytosols from adult rats (450 g b.w.) were performed as described previously (1, 2). Rete testis fluid was collected from rats (450 g b.w.) by micropuncture 4 h after ligation of efferent ducts while rats were anesthetized with sodium pentobarbital (40 mg/kg b.w., intraperitoneal) as described (15, 16). The use of animals for studies described in this report was approved by the Rockefeller University Institution Animal Care and Use Committee with Protocol Numbers 94–132, 95–129, 95–29-R1, and 97–117.

Preparation of Testicular Cell Cultures

Sertoli Cells Cultured at Low Density—Primary Sertoli cells were prepared from 20-day-old Sprague-Dawley rats as described previously (6, 8). All the experiments using Sertoli cell-enriched cultures reported in this paper were derived from 20-day-old rats unless otherwise specified. Cells were plated at a density of 4.5 × 10⁶ cells/ml/100-mm dish (about 5 × 10⁶ cells/cm²) in serum-free Ham’s F12 nutrient mixture/ Dulbecco’s modified Eagle’s medium (F12/DMEM, 1:1, v/v) supplemented with insulin (10⁻⁶ g/ml), human transferrin (5 μg/ml), bacteriostrept (5 μg/ml), and epidermal growth factor (5 ng/ml) and incubated at 35 °C in a humidified atmosphere of 95% air, 5% CO₂. About 48 h after plating, cultures were hypotonically treated with 20 mM Tris-HCl, pH 7.4, for 2.5 min to lyse residual germ cells (17) to obtain Sertoli cell cultures with greater than 95% purity. Cells were washed twice with F12/DMEM, and the cells were allowed to recover for an additional 24 h before their use. Under these conditions, specialized tight junctions were not formed when assessed by various criteria as described previously (4).

Sertoli Cells Cultured at High Density—To assess the effect of anti-testin IgG or [Ca²⁺] depletion on the inter-Sertoli tight junction, primary Sertoli cells isolated as described above from 20-day-old rats were cultured at high cell density to allow the establishment of specialized junctions. Briefly, about 2 × 10⁶ cells/cm² were plated on Matrigel™ (1.8)-coated HA filters in the apical chamber of a bicameral unit (Millipore, Bedford, MA) (4). To assess the formation of tight junctions, TER across the Sertoli cell monolayer was quantified using a Millicell electrical resistance system. Briefly, current was passed through the epithelial monolayer between two silver-silver chloride electrodes. Resistance was calculated from the change in voltage across the monolayer induced by a short (2–5 s) 20-μA pulse of current. The resistance was multiplied by the surface area of the filter to yield the areal resistance in ohms/cm². The net value of electrical resistance was then computed by subtracting the background, which was measured on Matrigel-coated cell-free chambers, from values of Sertoli cell-plated chambers. Disruption of the tight junctions were achieved by incubating the Sertoli cell monolayer in [Ca²⁺]-free F12/DMEM for 15 min as described (14), which is manifested by a drastic decline in TER. Thereafter, cells were returned to [Ca²⁺]-containing F12/DMEM with or without anti-testin IgG or normal rabbit serum IgG (200 μg/ml) for 20 min at room temperature with gentle rocking and then returned to 35 °C for the re-establishment of the tight junction as described previously (14). Disruption and reformation of the tight junction was assessed by TER measurement. IgG was purified from decolplemented sera (56 °C for 30 min) by sequential ammonium sulfate precipitation and DEAE chromatography as described previously (18). Each sample contained triplicate cultures, and each experiment was repeated at least three times using different batches of cells.

Primary Sertoli Cell Cultures from 35- and 90-day-old Rats—Primary cultures of Sertoli cells from 35- and 90-day-old Sprague-Dawley rats were prepared essentially as described previously (19). The cells were suspended in F12/DMEM supplemented with various factors as described for immature Sertoli cells (see above) and plated at approxi- mately 4.5 × 10⁶ cells/9 ml/100-mm dish and cultured for 2 days at 35 °C with 95% air, 5% CO₂. Thereafter, cells were hypotonically treated to remove the residual germ cells (17). The resulting cell purity was about 85% when judged microscopically, and these cells were used for RNA extraction after 4 days in culture to compare the basal steady-state testin mRNA level between Sertoli cells isolated from rats of different ages.

Germ Cells—Total germ cells were isolated from 90-day-old Sprague-Dawley rat (about 300 g b.w.) testes by a mechanical procedure without any enzymatic treatment as detailed elsewhere (20). These cell preparations consisted largely of spermatogonia, spermatocytes, and round spermatids with a relative percentage of 16:19:65 when verified by microscopic examination and DNA flow cytometry as described (20, 21). Virtually all elongated spermatids were removed in the glass wool filtration step (20). Germ cells were cultured at a density of 22.5 × 10⁶ cells/ml of F12/DMEM supplemented with sodium π-lactate (6 mM) and sodium pyruvate (2 mM) in 100-mm dishes for 20 h at 35 °C to obtain germ cell-conditioned medium (GCCM) as described (20, 21) or used immediately after their isolation for binding and coulture experiments. These cells were largely free of somatic cell contamination when assessed by various criteria as detailed elsewhere (20).

Sertoli-Germ Cell Cocultures—To assess the effects of germ cells on Sertoli cell terin expression, primary Sertoli cells isolated from 20-day-old rats were plated at 5 × 10⁶ cells/cm² and cultured for 2 days; thereafter, cells were hypotonically treated to remove contaminating germ cells (17) (day 0). Cells were then cultured for an additional 24 h (day 1), washed once, and cultured for 3 additional days (day 4) to ensure that the testin steady-state mRNA level had returned to base line since the culture procedure per se can induce damage on the intercellular junctions, which is a potent stimulator of testin expression (8). Thereafter, different amounts of germ cells using a Sertoli:germ cell ratio of 1:2.5, 1:5, and 1:10 were added to Sertoli cells and cultured for an additional 20 h (day 5). It must be noted that under these conditions, specialized junctions between Sertoli and germ cells had not yet formed, since their formation would require at least 24–48 h in culture (22, 23). Before their termination, cultures were subjected to a hypotonic treatment to lyse germ cells 20 min before the addition of RNA STAT-60™ for RNA extraction to eliminate RNA contributed by germ cells in the sample to be analyzed. As such, the RNA that were examined in this experiment were largely derived from Sertoli cells. To assess the effect of the disruption of Sertoli-germ cell junctions, some cocultures that had incubated for 30 h (day 5) were subjected to a hypotonic treatment on day 5 that was 24 h before their termination on day 6. Control dishes were Sertoli-germ cell cocultures incubated until day 6 without hypotonic treat. The samples were normalized after densitometric scanning analysis.

Pulse-Chase Analysis of the Synthesis and Secretion of Testin by Sertoli Cells in Sertoli-Germ Cell Cocultures

To assess the effect of germ cells or GCCM on the synthesis and secretion of testin by immature Sertoli cells in vitro, Sertoli cells were prepared at a density of 4.5 × 10⁶ cells/9 ml/100-mm dish as described above. Twenty-four h after the hypotonic treatment, germ cells (10 × 10⁶ cells/dish) or GCCM (10 μg of protein/dish) were added and cocultured with Sertoli cells for 18 h in F12/DMEM containing 1/100th of the normal methionine concentration. The cells were then pulse-labeled for 15 min with 100 μCi of [³⁵S]methionine per dish and subsequently washed three times to remove any remaining [³⁵S]methionine. At specified time points, media and cells were harvested and stored at −20 °C until used. Immunoprecipitation was performed as described (4). In one set of control experiments (Con 1), Sertoli cells were cultured alone without germ cells or GCCM and immunoprecipitated with the testin antibody at specified time points. In other controls (Con 2), Sertoli cells were cultured alone but immunoprecipitated with preimmune serum to assess the specificity of the testin antibody. Samples were resolved onto 10% T polyacrylamide gels and visualized by autoradiography.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from cell cultures or tissues using RNA STAT-60™ as described previously (6, 8, 24). Northern blot analysis was performed as described previously using a α-²⁸P-labeled testin cDNA probe by nick translation (6) for hybridization. To ensure that equal amounts of RNA were loaded into each lane, some blots were rehybridized with a α-²⁸P-labeled β-actin cDNA probe (6), and data were normalized after densitometric scanning analysis.
**Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)**

Recent studies have shown that glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression is increased in various cell types under oxidative stress, which is a common feature of aged cells. This enzyme plays a key role in glycolysis by catalyzing the conversion of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. It is also involved in the regulation of cell metabolism and has been implicated in cell signaling pathways, stress responses, and neurodegeneration. The increased expression of GAPDH under stress conditions suggests its potential as a biomarker for cellular stress and may have implications for understanding the molecular mechanisms underlying aging and disease.
the level of testin in rete testis fluid from intact adult testes was several orders of magnitude lower than these three other secretory proteins, which were highly concentrated in the rete testis fluid (Fig. 1B). By any measure, it is logical to expect that any secretory proteins of Sertoli cell origin be concentrated in the luminal fluid since the blood-testis barrier formed by adjacent Sertoli cells limits the transport of proteins from the systemic circulation to the interstitium unless it is being actively transported there. On the other hand, testin may be rapidly reabsorbed by either Sertoli or germ cells after its secretion by Sertoli cells. If testin is indeed secreted basally, it is expected to be detected in the serum at high concentration; however, earlier RIA data revealed a very low level of testin in the serum, and its concentration did not alter after orchiectomy (2). As such, results shown in Fig. 1B illustrate the unique feature of testin, indicating this protein is not accumulated in the luminal fluid and is likely to be reabsorbed onto the testicular cell. When the levels of testin in the cytosols of the intact testis (Fig. 1C) and epididymis (Fig. 1D) were quantified and compared with these other Sertoli cell proteins, it was found that testin was almost negligible in both organs when compared with these other Sertoli cell proteins, further suggesting that the secreted testin might become reabsorbed by testicular cells.

**Binding of 125I-Testin by Sertoli Cells, Germ Cells, and ECM**—To investigate whether testin is bound to the Sertoli cell membrane, the following experiments were performed. Highly purified Sertoli cells prepared as described under “Materials and Methods” were plated in 12-well dishes at 5 × 10^5 cells/well/ml F12/DMEM. Briefly, 125I-testin (1 × 10^6 cpm, about 10 fmol) was incubated with cells for specific time points at 35 or 4 °C in F12/DMEM (Fig. 2A). Nonspecific binding was estimated by using 100-fold excess unlabeled purified testin. Cells were then washed three times in F12/DMEM to remove unbound 125I-testin. Cells were subsequently solubilized in 1 M NaOH for radioactivity determination. Fig. 2A shows that Sertoli cells bound 125I-testin specifically in a temperature-dependent manner, which the binding was saturable by 2 h. We next investigated whether testin can also bind to germ cells or ECM. Sertoli cells were cultured at 4.5 × 10^6 cells/9 ml/100-mm dish for 2 days, containing germ cells were lysed by a hypotonic treatment (17), and the cultures were incubated at 35 °C for 4 days. Highly purified Sertoli cells or germ cells were then incubated with 125I-testin (about 5 × 10^6 cpm) for 4 h at 35 °C. The dishes were washed, and cell membranes were isolated as described under “Materials and Methods.” Cell membranes and ECM were then solubilized in the corresponding buffer, heated at 100 °C for 10 min, and resolved by SDS-PAGE, and the 125I-testin was visualized by autoradiography. In Fig. 2B, lanes 1 and 10 are the 125I-testin used for incubation, showing the two molecular variants of testin I and testin II. Lanes 2–5 are the Sertoli cell membranes solubilized with 20 mM Tris containing 1.6% 2-mercaptoethanol; 20 mM Tris containing 1% SDS; 20 mM Tris containing 1% SDS and 1.6% 2-mercaptoethanol; and 20 mM Tris containing 1% SDS, 1% Triton X-100, 1% Nonidet P-40, and 1.6% 2-mercaptoethanol, respectively. All buffers used were adjusted to pH 6.8 at 22 °C. Lanes 6–9 are the ECM on the Petri dish extracted with the same buffers as shown in lanes 2–5. These results indicate not only that 125I-testin binds onto the Sertoli cell membrane and ECM, but its solubilization requires the use of detergents, suggesting that its association with the cell membrane is not a nonspecific attachment. In the absence of detergents (lanes 2 and 6 versus lanes 3–5 and 7–9, Fig. 2B), none of the 125I-testin that was bound onto the Sertoli cells could be solubilized. Germ cells also bound 125I-testin (lane 11, Fig. 2B) specifically, since the presence of 100-fold excess unlabeled testin (lane 12, Fig. 2B) competed with the binding.

**Identification of a Protein Complex on the Sertoli Cell Membrane That Binds Testin**—We next sought to investigate whether the binding of 125I-testin onto the cell membrane as shown in Fig. 2 is mediated by a binding protein. Sertoli cells were cultured at high cell density on Matrigel-coated dishes for 4 days to allow the formation of specialized junctions. Plasma membranes were isolated as described under “Materials and Methods,” and the whole membrane fraction was labeled with Na[125I] by iodogen (26). Immunoprecipitation was then performed on the labeled membrane proteins using either testin antibody or preimmune serum (1:50). The monospecificity of this antiserum has been characterized and established (2, 4, 6). Immunocomplexes were purified by protein A-Sepharose column and resolved on a SDS-polyacrylamide gel and shown in Fig. 3A. Lane 1 (S) is the 14C-methylated protein standard. Lane 3 is 125I-testin, where the two molecular weight variants of testin were clearly visible. Lanes 2 and 4 are the Sertoli cell membrane (SM) proteins after cell labeling and immunoprecipitation run under reducing (R) and nonreducing (NR) conditions, respectively. Radiolabeled testin I and II extracted from the membrane are clearly visible in the SM samples (Fig. 3A, lanes 2 and 4) when compared with 125I-testin alone (lane 3). It was noted that two other cell membrane proteins that were labeled and immunoprecipitated in conjunction with testin, designated as a (45 kDa) and b (28 kDa) are also visible in lanes 2 (reducing) and 4 (nonreducing), indicating that they are single polypeptide chains. Lanes 5 and 6 are the corresponding controls of lanes 2 and 4, where the testin antibody incubation was substituted with preimmune serum, illustrating the bands a and b shown in Fig. 3A are components of the binding protein complex. These results, however, cannot distinguish whether these two peptides reside on the cell surface or are found inside the membrane. In contrast to the whole membrane labeling, Fig. 3B shows the result obtained from the surface labeling of viable Sertoli cells previously treated with a membrane-impermeable and thiol-cleavable cross-linker, DTSSP. Lane 1 (S) is the 14C-methylated protein standard. Lane 2 is 125I-testin, where the two molecular variants of testin are clearly visible. Lanes 3 and 4 are the Sertoli cell surface-labeled proteins after extraction and immunoprecipitation run under reducing (R) and nonreducing (NR) conditions, respectively. Radiolabeled testin I and II in conjunction with the 28-kDa protein, desig-
nated b, are clearly visible in the DTSSP-treated surface-labeled sample (Fig. 3B, Lane 3) under reducing conditions. Under nonreducing conditions, the band b, together with testin I and II, were not visible and possibly retarded in the gel because the complex had not been cleaved with a reducing agent, since DTSSP is a thiol-cleavable cross-linker (Fig. 3B, lane 4 versus lane 3). These results indicate that the 28-kDa protein (band b) and testin I and II reside on the cell surface, and the 45-kDa protein a is localized inside the cell membrane. In both sets of experiments, the gels were also Coomassie Blue-stained to visualize any proteins that might not have been labeled. However, no other proteins were identified.

Localization of Testin in the Rat Testis by Immunogold EM—To examine the subcellular localization of testin, immunogold EM was carried out, and about 300 cross-sections were examined. Fig. 4 summarizes the result of these analyses. Immunoreactive testin as marked by gold (black) particles was abundantly localized near the surface of a Sertoli cell adjacent to the ectoplasmic specialization (a modified adherens junction) around a developing spermatid (Fig. 4A). The Sertoli cell was typified by the presence of microtubule bundles (Fig. 4A, arrowheads). Very few gold particles representing immunoreactive testin were found along the tight junction between two adjacent Sertoli cells (Fig. 4B). These data are in agreement with previously published immunofluorescent microscopy and immunohistochemistry revealing the abundant presence of testin between Sertoli and germ cells (5–7), Fig. 4C is a control section stained with IgG isolated from preimmune serum showing a cross-section between two Sertoli cells as typified by the presence of actin filament bundles (shown by the arrowheads) where no gold particles were seen. The plasma membranes may not be obvious in these sections, probably due to the omission of OsO4 in the initial tissue fixation, since the use of this fixative caused a loss of antigenicity of the testin molecule, making localization impossible.

Effect of Anti-testin IgG on the Formation of Tight Junctions and the Expression of Testin after Their Disruption by Calcium Depletion—Since earlier studies reveal a tight relationship between the expression of testin and the integrity of intercellular cell junctions in the testis (8), we sought to examine whether a disruption of the inter-Sertoli tight junction can induce a surge in testin expression similar to what was shown when the Sertoli-germ cell junctions were disrupted (8). It is known that epithelial tight junctions, such as those found in the Madin-Darby canine kidney cells in vitro, can be disrupted by the removal of calcium ions from the medium and can be quickly reassembled after its replacement (14, 33, 34). The integrity of the inter-Sertoli tight junctions was assessed by TER measurement (31, 32). The inter-Sertoli tight junction was disrupted by [Ca2+] depletion in primary Sertoli cell cultures. Sertoli cell cultures were prepared as described under “Materials and Methods,” and TER measurements were taken thereafter. It was noted that tight junctions were established in these Sertoli cell cultures by day 3–4 (Fig. 5A), when the TER reached its plateau with a measurement of about 50–60 ohms/cm ², which is similar to a previous report (35). On day 5, extracellular [Ca²⁺] was removed from the bicameral unit by rinsing the cell monolayer gently with Ca²⁺-free F12/DMEM, and the units were incubated in Ca²⁺-free F12/DMEM for 15 min. At the end of this 15-min period, a significant decline in TER was noted (Fig. 5A), illustrating the tight junction had become leaky. This result is consistent with other tight junction forming epithelia such as Madin-Darby canine kidney cells (14). In some experiments, cells were incubated with or without either anti-testin IgG or anti-clusterin IgG.
IgG (200 μg/ml) or normal rabbit IgG (200 μg/ml) together with fresh F12/DMEM containing [Ca^{2+}] to assess the effect of testin antibody on the reformation of tight junctions between Sertoli cells. It was noted that the tight junctions were re-established within 90 min (Fig. 5A) as shown by an increase in TER. However, the presence of anti-testin IgG did not interfere with the reformation of tight junctions in these cultures (Fig. 5A). More important, when the testin steady-state mRNA level was assessed in the samples when the tight junctions were being disrupted and re-established, no detectable change in testin expression was noted (Fig. 5B). When the same blot shown in Fig. 5B was rehybridized with a β-actin probe (Fig. 5C) and the data were densitometrically scanned and normalized against β-actin (Fig. 5D), testin expression was found not to correlate with the disruption or formation of tight junction, which is entirely different from the testin mRNA expression

**FIG. 2.** Binding of {sup 125}I-testin to primary Sertoli cell cultures (A), and its solubilization from Sertoli cells (SC), germ cells (GC), and ECM (B). A, binding assay was performed as described under “Materials and Methods.” {sup 125}I-Testin (1 × 10⁶ cpm) was incubated at 35 or 4 °C for specified time points. Nonspecific binding was estimated using a 100-fold excess of unlabeled testin. B, autoradiograph showing the solubilization of membrane-bound {sup 125}I-testin from SC, GC, and ECM. {sup 125}I-Testin (5 × 10⁶ cpm/5 ml/100-mm dish) was incubated with primary Sertoli cells for 4 h at 35 °C as described under “Materials and Methods.” Membrane proteins were solubilized in 100 μl of corresponding buffer, heated at 100 °C for 10 min, and resolved on a 10% T SDS-polyacrylamide gel, and testin was visualized by autoradiography. Lanes 1 and 10, {sup 125}I-testin tracer. Lanes 2–5, SC membranes solubilized with either 20 mM Tris containing 1.6% 2-mercaptoethanol, 20 mM Tris containing 1% SDS and 1.6% 2-mercaptoethanol, 20 mM Tris containing 1% SDS, or 20 mM Tris containing 1% SDS, 1% Triton X-100, 1% Nonidet P-40, and 1.6% 2-mercaptoethanol, respectively. All buffers were adjusted to pH 6.8 at 22 °C. Lanes 6–9 is the remaining ECM on the SC dishes extracted with the same buffers as shown in lanes 2–5. Primary adult germ cells were also incubated for 4 h at 35 °C with {sup 125}I-testin (lane 11) or with {sup 125}I-testin plus a 100-fold excess of unlabeled testin to assess nonspecific binding (lane 12). The germ cell membranes were solubilized in SDS sample buffer and heated at 100 °C for 10 min.

**FIG. 3.** Identification of the testin binding protein complex on the Sertoli cell membrane. Proteins were visualized by SDS-PAGE and autoradiography under reducing (R) and nonreducing (NR) conditions. A, cell membrane proteins labeled with Na{sup 125}I were visualized by SDS-PAGE and autoradiography after immunoprecipitation run under reducing and nonreducing conditions, respectively. Radiolabeled testin I and II extracted from the membrane are seen in the Sertoli cell membrane samples along with the p(28 kDa) peptide (lane 3), which was retarded in the gel in the absence of reducing agent (2-mercaptoethanol) (lane 4). These results illustrate that testin I, II, and the b peptide reside on the Sertoli cell surface.
shown that glycerol can induce permanent damage of the blood-testis barrier by disrupting the inter-Sertoli tight junction near the basal lamina within 2 weeks after glycerol administration (25). A disruption of the blood-testis barrier was manifested by an influx of $[^{3}H]$inulin and $[^{125}I]$albumin to the rete testis fluid, seminiferous tubule fluid, and the testicular tissue within 2 weeks post-glycerol treatment after in vivo infusion of radiolabeled substances (25). We have examined the effect of glycerol-induced disruption of inter-Sertoli tight junctions and the subsequent damage on Sertoli-germ cell junctions as a result of germ cell depletion on the steady-state testicular testin mRNA level (Fig. 6). The morphological changes in the testis after glycerol treatment were also examined immunohistochemically where testin appears as a reddish-brown precipitate (Fig. 7). Adult rats were anesthetized with Metofane and received control (PBS) or treatment solution (200μl of PBS containing 20% glycerol) injected via a 26-gauge needle through the polar axis of each testis beginning at one pole and terminating at the other (25). 2, 4, and 8 weeks later, testes were removed for RNA extraction and Northern blot analysis. Fig. 6A is a Northern blot using about 20μg of total RNA per lane. No changes in the testicular testin steady-state mRNA level were apparent by 2 weeks at the time when tight junctions were disrupted (Fig. 6, A and B). It was found that virtually all germ cells were still present in the epithelium by 2 weeks after glycerol administration (Fig. 7C versus Fig. 7, A and B). However, a significant increase in the testin steady-state mRNA level (Fig. 6, A and B), which was also accompanied by an accumulation of testin (Fig. 6, C and D) in the testis was clearly visible by 4 and 8 weeks after glycerol treatment when germ cells, in particular round and elongated spermatids, were depleted from the seminiferous epithelium at 4–8 weeks (Fig. 7D and E versus Fig. 7, A, B, and C). When the concentrations of testin in the testes of these rats were quantified by RIA and compared with control rats, a significant increase in testin concentration was noted (Fig. 6C). Since there was a significant decline in testicular weight by 8 weeks after glycerol treatment, the changes in testin level were taken into account with the reduction in testicular weight. Once the data were expressed as testin per testes (Fig. 6D), a 7-fold increase was detected in glycerol-treated rats by 8 weeks. These results are also consistent with the immunohistochemistry data, since there is drastic increase in immunoactive testin accumulated in the tubular lumen at 4–8 weeks after glycerol treatment (Fig. 7, D and E) compared with control rats (Fig. 7B) and rats treated with glycerol for 2 weeks (Fig. 7C). Glycerol, however, had no effect on testin expression in the epididymis up to 8 weeks post-treatment (Fig. 6, A and B), illustrating the specificity of this chemical treatment in the testis. However, immunohistochemistry analysis revealed that testin was accumulated in the epididymal lumen 4–8 weeks post-glycerol treatment (data not shown).

Effect of GCCM or Germ Cells on Sertoli Cell Testin Steady-state mRNA Level—It is known that germ cells neither express testin mRNA (7) nor do they secrete any testin in vitro (20). But it is not known whether GCCM or germ cells can regulate testin expression. To examine such a possibility, Sertoli cells cultured at $5 \times 10^{5}$ cells/cm$^2$ when specialized junctions did not form were incubated with increasing concentrations of GCCM proteins for a 20-h period (Fig. 8A). Fig. 8A shows that GCCM had no affect on the Sertoli cell testin steady-state mRNA level. Fig. 8B is the same blot such as the one shown in Fig. 8A but hybridized with a β-actin cDNA probe. Fig. 8C is the densitometrically scanned data of three separate Northern blots normalized against β-actin, indicating that germ cell-released proteins had no apparent effect on Sertoli cell testin expression. Fig. 8D is the RIA result showing the concentration of testin in...
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We next examined whether germ cells can affect Sertoli cell testin expression in vitro (Fig. 9). Since the isolation of Sertoli cells from the seminiferous tubule involves the disruption of Sertoli-germ cell junctions, which would have induced changes in testin expression, Sertoli cells used for this experiment were cultured for 4 days before the addition of germ cells to allow its testin steady-state mRNA level to return to the basal level. Lane 1 is total RNA derived from Sertoli cells cultured for 48 h in vitro; cells were terminated 20 min before the hypotonic treatment (Day 0). Lane 2 is RNA from Sertoli cell cultures 24 h after hypotonic treatment (Day 1). Lane 3 is the Sertoli cell RNA isolated from cultures on day 5 that served as a control for lanes 4–6. As expected, a steady decline in testin expression during cultures is noted (Fig. 9A, lanes 1–3), suggesting that the isolation of Sertoli cells from the seminiferous tubule can enhance testin expression because these steps disrupted the Sertoli-germ cell junctions. The hypotonic treatment step on day 0 (i.e. 48 h after Sertoli cells were isolated from the tubules) could not induce a surge in testin expression in these cultures (Fig. 9A, lane 2 versus lane 1) possibly because these primary cultures were relatively free of germ cells; as such, not many Sertoli-germ cell junctions could be disrupted. Alternatively, the expression of testin at this time was already maximized, and the removal of residual germ cells could no longer elicit an additional increase in testin expression. Different germ cell numbers using a Sertoli:germ cell ratio of 1:2.5, 1:5, and 1:10 were then plated onto these Sertoli cell cultures on day 4 and incubated for an additional 20 h (day 5) to examine the effects of germ cells. Immediately before RNA extraction, each culture dish was hypotonically treated to lyse germ cells to eliminate RNA contributed by germ cells in the sample to be analyzed. As such, total RNA extracted from these dishes were largely of Sertoli cell origin. It was found that germ cells did not generate a dose-dependent and significant effect on the Sertoli cell testin expression as shown in Fig. 9, A–C (lanes 4–6 versus lane 3), even though it is apparent that the presence of germ cells reduced Sertoli cell testin expression slightly. It must be noted that under these conditions, specialized Sertoli-germ cell junctions did not form since their formation would require an incubation period of at least 24–48 h in vitro (22, 23). In some experiments, the Sertoli-germ cell cocultures were allowed to incubate for 30 h in vitro and were subjected to a hypotonic treatment on day 5 to disrupt the Sertoli-germ cell junctions, and the cells were harvested on day 6 for analysis. A significant increase in testin expression was found as a result of the disruption of the junctions (Fig. 9A, lane 7 versus lane 8).

Effect of Germ Cells or GCCM on Testin Synthesis and Secretion—To study the effect of germ cells or GCCM on Sertoli cell testin secretion, Sertoli cells (4.5 × 10⁶ cells/9 ml/100-mm dish) were cocultured with germ cells (1 × 10⁵) or GCCM (10 μg of protein) for 18 h in vitro. Thereafter, cells were pulse-labeled with [35S]methionine for 15 min and chased with cold methionine at specified time points. Fig. 10, A and B show the relative amounts of [35S]-labeled testin in the cytosol and media, respectively, at specified time points. It was noted that testin appeared in the cytosol within 15 min with a testin/18S ratio of 3:1 when the x-ray film was densitometrically scanned.
at 600 nm (Fig. 10A). By 24 h, all the newly synthesized $^{35}$S-labeled testin was no longer detectable in the cytosol but secreted into the medium (Fig. 10A), indicating that germ cells did not inhibit testin secretion into the media. Testin was detected in the medium within 1 h after pulse-labeling and peaked at 24 h, concomitant with the disappearance of testin in the cytosol, but the ratio of testin I:testin II had shifted, becoming 1:1.5 (Fig. 10B versus A). The reason for such a shift in ratio is not immediately known; it is possibly the result of post-translational processing such as glycosylation. An age-dependent reduction of testin expression likely correlates with the onset of spermatogenesis. We next examined the changes in testin steady-state mRNA level in the testis during maturation. When the changes in testin steady-state mRNA level in the testis were quantified by Northern blot, it was found that tubules from immature rats had almost 10-fold more testin mRNA than mature rats (8). However, these earlier studies did not take into consideration the increase in RNA contributed by germ cells in the samples being analyzed, since there is a drastic increase in germ cell:Sertoli cell ratio during maturation. When the changes in testin steady-state mRNA level in the testes were compared between Sertoli cells isolated from 20- and 90-day-old rats (Fig. 12A), it was noted when about 10 $\mu$g of total RNA was used for analysis (Fig. 12B). These blots were then rehybridized with a $\beta$-actin probe and densitometrically scanned at 600 nm, and the data were normalized against $\beta$-actin. An 8-fold reduction in testin expression was observed during maturation, when the steady-state mRNA level was compared between Sertoli cells isolated from 20- and 90-day-old rats (Fig. 12C). These results illustrate that an age-dependent reduction in testin expression likely correlates with the differentiation status of the Sertoli cell.

DISCUSSION

Table I summarizes the three types of specialized junctions that are present in other epithelia that are also found in the mammalian testis (for reviews, see Refs. 11–13). Three types of junctions, namely occluding, anchoring, and communicating junctions, can be found between Sertoli cells. However, only anchoring and communicating junctions are present between Sertoli and germ cells. Moreover, some of the anchoring junctions between Sertoli and germ cells such as tubulobulbar complexes are unique to the testis (Table I). Surprisingly, the component proteins that constitute these junctions between Sertoli and germ cells, let alone the molecules that regulate the disassembly and reassembly of these junctions throughout different stages of spermatogenesis as a result of migration of developing germ cells from the basal lamina to the adluminal compartment of the seminiferous epithelium (for reviews, see Refs. 11, 12), are largely unknown. Although the mechanisms and cellular events that regulate germ cell translocation in the epithelium are poorly understood, studies in organogenesis, embryogenesis, tumor growth, and metastasis have yielded some crucial information with regard to germ cell movement, since these other cellular processes also involve extensive turnover of cell-cell and cell-matrix interactions as well as cell migration (for reviews, see Refs. 38 and 39). It is known that
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Both embryogenesis and tumor growth involve the participation of proteases, protease inhibitors, signaling molecules, growth factors, and junctional complex components such as cell adhesion molecules (for reviews, see Refs. 40–45). For the past decade, studies from different laboratories have identified several of these component molecules in the testis (for reviews, see Refs. 11–13 and 46). Recent studies from this laboratory have also illustrated the involvement of proteases, protease inhibitors, and cell adhesion molecules in the early stage of Sertoli-germ cell interactions preceding the establishment of specialized cell junctions in vitro (47, 48), suggesting that the establishment of specialized junctions between testicular cells is not simply a series of morphological events. Instead, multiple factors are involved, illustrating the complexity of junction formation in the seminiferous epithelium. The present report demonstrates testin is likely to be a sensitive marker to probe the events of cell junction disruption in the testis.

In this study, testin was shown to correlate with the disruption of Sertoli-germ cell junctions but not the inter-Sertoli tight junction. Several lines of evidence have excluded the involvement of testin in the formation and/or disruption of tight junction. First, immunogold EM revealed very few testin are found in the tight junction between Sertoli cells. Second, anti-testin IgG did not interfere or facilitate the formation of tight junctions between Sertoli cells in vitro, nor did it affect the recovery of tight junctions after [Ca<sup>2+</sup>] depletion induced disruption of the inter-Sertoli tight junction. Third, the expression of testin is not affected by the disruption of tight junctions in vitro, as demonstrated in the [Ca<sup>2+</sup>] depletion experiment. This observation is in sharp contrast to the disruption of Sertoli-germ cell junctions, which is accompanied by a surge in testin expression (8). Fourth, changes in testin expression after glycerol treatment did not coincide with the damage of the blood-testis barrier by 2 weeks (25) but rather, with the depletion of germ cells, which disrupted the Sertoli-germ cell junctions. We thus conclude that the testin that was found in both the adluminal and basal compartments of the seminiferous epithelium as visualized by immunofluorescent microscopy and immunohistochemistry (5–7) is the protein localized between Sertoli and germ cells, most likely at the adherens junction such as the desmosome-like junction, ectoplasmic specialization, and tubulolubar complex. It is unlikely that testin is involved in the gap junction, since the component molecules of the gap junction have been very well characterized, and the primary sequence of testin does not bear any homology to any of the existing connexin family members (for review, see Ref. 13).

It has been shown that the testin steady-state mRNA level in the adult rat testis is significantly enhanced as a result of either a chemical treatment such as glycerol, lonidamine (8), and busulfan (3) or a physical treatment such as hypotonic treatment (8) and X-irradiation (10). The depletion of germ cells after these treatments would undoubtedly disrupt the Sertoli-germ cell junctions. It is our belief that several factors may be operating independently or synergistically that regulate testin expression as shown in these in vitro and in vivo experiments. First, germ cells may regulate testin expression via cell-cell contacts or through a factor(s) released from germ cells. As such, a depletion of germ cells leads to a change in testin expression. Second, testin may be a structural component of the Sertoli-germ cell junction. Thus, when the intercellular junction is damaged, another yet-to-be identified factor is released to stimulate the production of testin to replace the lost structural component or to trigger another cascade of events. Third, testin may be a stress-induced protein in response to the

FIG. 7. Morphological changes and the associated pattern of testin immunohistochemical localization in adult rat testes after intratesticular glycerol treatment. Immunoreactive testin appears as a reddish-brown precipitate as denoted by arrowhead. A and B are photomicrographs of the cryostat sections of testes from a control rat. C, D, and E are photomicrographs of rats treated with glycerol after 2, 4, and 8 weeks, respectively. Four animals in each treatment group including control were processed for microscopic examination, and at least 50–100 sections were examined. A single set of representative data are shown here. A, control rat showing the cross-section of a normal seminiferous tubule at stage VII of the spermatogenic cycle stained with testin antibody. Immunoreactive testin was found between spermatocytes/spermatogonia and Sertoli cells at the basal compartment. Testin was also found between Sertoli cells and the heads of the elongated spermatids (es) at the adluminal compartment. C, two weeks after glycerol treatment, it is noted that all elongated spermatids were depleted, but the number of round spermatids (rs) and spermatocytes (p) remained relatively unchanged. Testin is still localized in the basal compartment between Sertoli and germ cells. D and eight (E) weeks after glycerol treatment, a massive reduction of germ cells from the epithelium and an accumulation of immunoreactive testin was found in the lumen of the tubule.
stress and/or cellular death caused by these experimental manipulations.

The present study using germ cells or their conditioned medium cocultured with Sertoli cells failed to demonstrate a significant effect on Sertoli cell testin steady-state mRNA level. This result seemingly suggests that germ cells do not play a major role in regulating testin expression. However, one must note that these cocultures were terminated at 20 h, at the time when specialized Sertoli-germ cell junctions had not yet formed, since morphological analysis has shown that the establishment of specialized junctions between Sertoli and germ cells in vitro, such as desmosome-like adherens junctions, requires a culture period of 24–48 h (22, 23). As such, it remains to be determined whether testin is involved in the formation of Sertoli-germ cell junctions. The fact that there is a surge in testin expression when the Sertoli-germ cell junctions are disrupted may suggest testin somehow participates in the destruc-

FIG. 8. Effects of GCCM on the Sertoli cell steady-state testin mRNA level and the amount of testin secreted by Sertoli cells in vitro. A, Northern blot showing the level of Sertoli cell testin expression when cultured with an increasing concentration of GCCM for a 20-h period. Approximately 10 μg of total RNA were loaded per lane, kb, kilobases. B, the same blot shown in A but rehybridized with a β-actin probe. C, a graph showing the relative testin mRNA level in Sertoli cells cultured with various amounts of GCCM proteins and normalized against β-actin after densitometric scanning of three blots such as the one shown in A. D, the concentration of testin in the spent medium in these cultures was quantified by a testin-specific RIA. ns, not significantly different from Sertoli cells cultured in the absence of germ cells; *, p < 0.01.

FIG. 9. The effect of germ cells (GC) and hypotonic treatment (HT) on the SC testin steady-state mRNA level in Sertoli-germ cell cocultures in vitro. A, Northern blot showing the steady-state testin mRNA level in Sertoli cells when cocultured with increasing numbers of germ cells for a 20-h period. Approximately 10 μg of total RNA were loaded per lane. In these cultures, germ cells were lysed by a hypotonic treatment 20 min before their termination to eliminate RNA contributed by germ cells in the samples being analyzed. kb, kilobases. B, ethidium bromide staining of the same blot shown in A. C, a graph showing the relative testin mRNA level in Sertoli cells cocultured with germ cells after densitometric scanning at 600 nm of three Northerns such as the one shown in A. BH, Sertoli cells were terminated 20 min before hypotonic treatment; AH, 24 h after hypotonic treatment; Ctrl, control cultures where Sertoli cells were cultured alone and terminated on day 5; 2.5, 5, and 10 are coculture experiments where germ cell:Sertoli cell ratio was at 2.5:1, 5:1, and 10:1; germ cells were added onto Sertoli cells (5 × 10^5 cells/cm^2) on day 4 and cocultured for 20 h and terminated on day 5; 10 H and Ctrl/10H correspond to lanes 7 and 8 shown in A.
tion of cell junctions by acting as a protease or that it protects the testis against tissue damage by acting as a protease inhibitor. Alternatively, the increase in testin expression is the result of cell junction disruption that is associated with other cellular events. However, testin was found to be neither a protease nor a protease inhibitor (6), making the former possibility unlikely. Although the glycerol or lonidamine (8)-induced Sertoli-germ cell junction damage can elicit an increase in testin accumulation in the testicular cytosol by up to 20–30-fold, most of the testin was accumulated in the lumen and was likely flushed out. Therefore, it is very unlikely that testin is being used for the reassembly of the damaged junction. The present study, however, illustrates that the testin steady-state mRNA level per pair of testes increases steadily during testicular maturation when the increase in organ weight and the increase in germ cell to Sertoli cell ratio are taken into consideration. These results suggest that the rapid assembly and disassembly of intercellular junctions at the onset of spermatogenesis is likely to be one of the most critical factors in regulating testin expression.

Studies on the binding of testin onto the Sertoli and germ cell membrane reveal that the dissociation of testin from the cell surface after its binding requires the use of a detergent illustrating that other biochemical changes may take place when testin couples onto the binding protein complex. This result also supports the notion that testin may be a structural component between Sertoli and germ cells. The amount of testin binding to Sertoli cells is limited to only 0.1–0.5% that of total 125I-testin in the incubation mixture, regardless of an increase in Sertoli cell number used for the in vitro assay. This result together with the detergent solubilization experiment strongly suggests that the interaction of testin with its binding protein complex is not a classical ligand-receptor interaction. The fact that a secretory protein can become tightly associated with the cell surface via a receptor-binding protein in a fashion dissimilar to a classical ligand-receptor interaction is not without precedence. Wnt, a growing class of multi-functional signaling molecules (glycoproteins with apparent Mr between 34,000 and 42,000) involved in both tumorigenesis and patterning events during development and tissue differentiation by coordinating the organization of groups of cells in the developing vertebrate (49, 50), are secretory proteins that can become tightly associated with the cell surface or ECM and originally thought to be via a nonreceptor-mediated mechanism (51–53). A recent study, however, has demonstrated that Dfz2, a 694-amino acid polypeptide, is the receptor protein of the signaling molecules of the Wnt gene family (54). The binding of the Wnt proteins onto the receptor, similar to testin, cannot be assessed by conventional receptor-ligand assays such as a Scatchard plot, probably due to their low abundance. A comparison between testin and nine members of the Wnt family, including Wnt-1 and Wnt-2 (55), using DNASIS, PROSIS, and BESTFIT programs at the levels of amino acid and nucleotide sequences revealed that they share 10–30% identity (data not shown). As such, testin is likely a distant member of the Wnt gene family, and it does share some of the unusual feature of the Wnt.

In the present and other earlier studies (3, 8, 10), it was shown that disruption of testicular cell junctions by glycerol, lonidamine, busulfan, or hypotonic treatment can lead to a surge in testin expression. Such a change in expression may be a response to cellular stress, when junctions are being disrupted. Studies in muscle, in particular, cardiac and skeletal myocytes, reveal that 5–30% of the cell population undergo plasma membrane disruption under physiological conditions, due to the contractile nature of these cells (56–58). Likewise,
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Fig. 12. The testin steady-state mRNA level in Sertoli cells isolated from rats at 20, 35, and 90 days of age, which illustrates an age-dependent reduction in expression. A, about 10 μg of total RNA was used per lane, and the Northern blot was hybridized with a 288-base pair testin cDNA probe as described under “Materials and Methods.” B, the same blot such as the one shown in A but stained with ethidium bromide, showing the integrity of the RNA; the same amount of RNA was used per each lane. C, densitometric scanning data of two blots such as the one shown in A at 600 nm but normalized against β-actin. D, day.

about 3% and 6% of the epidermal and endothelial cells in the skin (56) and aorta (59), respectively, are being disrupted. Even though the level of cell wounding for the Sertoli and germ cell in the testis is not known, extensive plasma membrane disruption in testicular cells is expected to occur due to the rapid morphological changes during spermatogenesis. Until recently, membrane resealing was thought to be a passive event. The “wound hormone” hypothesis (60) suggests that chemical mediators of tissue restructuring such as growth factors, stored in cytosol, are released during membrane disruptions (for review, see Ref. 61). For instance, basic fibroblast growth factor (bFGF), which lacks a signal peptide sequence, is a potent growth-promoting factor when it is released extracellularly due to plasma membrane damage (62, 63). Studies by immunoprecipitation have demonstrated the release of bFGF by germ cells (64), suggesting the bFGF found in GCCM is likely the result of germ cell wounding, since bFGF under the normal physiological condition is not a secretory protein because of the lack of a signal peptide. Other studies have demonstrated that the use of trypsin to isolate germ cells from the tubules (64) can alter many of the cell surface properties (20, 22, 65). As such, the secretion of bFGF by germ cells as demonstrated in this earlier study (64) may be the result of trypsin-induced plasma membrane damage that causes release of bFGF. It is possible that bFGF is an important growth-promoting and signaling molecule that participates in repairing the disrupted testicular cell junctions during spermatogenesis and whose release from germ cells is the result of degeneration (66–68) and apoptosis (69). Testin, on the other hand, is a secretory protein with a definite signal sequence (6). However, its protein level in the rete testis fluid and cytosols of the testis and epididymis as well as its expression are virtually undetectable except in the gonad. When Sertoli cells are being cultured in vitro, the amount of testin secreted into the spent medium is comparable with other Sertoli cell secretory products. It is our belief that upon dissociation of the testicular cells from the tubule during the preparation of Sertoli cells, testin expression is induced and remains elevated, as demonstrated in the present study. This observation thus supports the postulate that the expression of testin may be induced by cell wounding.

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