

Identification of Rat Testis Galactosyl Receptor Using Antibodies to Liver Asialoglycoprotein Receptor: Purification and Localization on Surfaces of Spermatogenic Cells and Sperm

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Abstract. We have found that the rat testis contains a cell surface galactosyl receptor that is antigenically related to the minor species of rat liver asialoglycoprotein receptor (ASGP-r) and has binding affinity for galactose coupled to agarose. In immunoblotting experiments, rat testis galactosyl receptor (RTG-r) is recognized by antiserum raised against the minor ASGP-r species of rat liver (designated rat hepatic lectin-2/3, RHL-2/3). Antiserum raised against the major species RHL-1 does not recognize an antigenic protein equivalent to RTG-r. Triton X-100-extracted rat liver and testes preparations fractionated by affinity chromatography on galactose-agarose and resolved by SDS-PAGE under reducing conditions, show that rat liver contains both the major (RHL-1) and minor (RHL-2/3) ASGP-r species whereas rat testis displays only a receptor spe-

cies comigrating with RHL-2/3. RTG-r was present throughout testicular development. The receptor was found in seminiferous tubules, cultured Sertoli and spermatogenic cells, and epididymal sperm. Indirect immunofluorescent studies show RHL-2/3-like immunoreactivity on the surface of Sertoli cell, meiotic prophase spermatocytes, spermatids, and epididymal sperm. In spermatids and sperm, the immunoreactivity is restricted to the plasma membrane overlying the dorsal portion of the head. Because the RTG-r has galactose binding affinity, is present on surfaces of Sertoli and developing meiotic and postmeiotic spermatogenic cells, and overlies a region of the intact acrosome on epididymal sperm, RTG-r may have a role in spermatogenesis and in events leading to sperm-egg recognition.

ONE feature of spermatogenesis and sperm maturation is the temporal appearance of cell-surface antigens organized into topographic domains (see reviews by Koheler, 1978; Millette, 1979; Holt, 1982). Some of these antigens are intrinsic membrane proteins persisting throughout spermatogenesis and sperm maturation and may have a role in zona pellucida penetration at fertilization (O'Rand et al., 1984). One of these membrane antigens is a functional sperm surface receptor with binding affinity for the egg zona pellucida (Shur and Hall, 1982).

Three categories of spermatogenic cell and sperm surface glycoprotein antigens have been identified according to their temporal expression: antigens expressed (a) during meiotic stages of spermatogenesis (mouse, Millette and Bellvé, 1977; rabbit, O'Rand and Romrell, 1977; rat, Tung and Fritz, 1978), (b) during spermiogenesis (guinea pig, Tung et al.,

1979; LeBouteiller et al., 1979), and (c) determinants appearing on sperm surface during epididymal transit (mouse, Feuchter et al., 1981). Some surface antigens are selectively partitioned during late spermiogenesis and are not detected on sperm after spermiation (Millette and Bellvé, 1977). The regionalized distribution of sperm surface components has been established with surface probes such as lectins specific to a variety of monosaccharides (Koehler, 1978), antibodies (Koehler et al., 1975; Bechtol et al., 1979), and covalent radiolabeling agents (Gabel et al., 1979). Freeze-fracture studies of sperm membranes have shown a remarkable regional distribution of intramembranous particles in sperm cell membrane (Friend and Fawcett, 1974) with changes related to sperm prefertilization events (Friend and Rudolf, 1974).

We have searched for cell surface receptors in spermatogenic cells which may bind and internalize testicular or extratesticular glycoproteins. This search was stimulated by our previous finding of binding and internalization of fetuin by rat Sertoli and spermatogenic cells upon co-culture in medium supplemented with fetal bovine serum (Abdullah et al., 1986). This finding suggested that testicular cells may have a receptor with functional characteristics of the asialo-

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glycoprotein receptor (ASGP-r),¹ an integral cell membrane glycoprotein relatively abundant in hepatocytes of several species (Ashwell and Morell, 1974; Ashwell and Harford, 1982). The identification of a testicular receptor with properties of ASGP-r has not been reported previously.

In hepatocytes, the ASGP-r mediates the internalization of various serum glycoproteins after removal of sialic acid residues from N-linked oligosaccharide chains (for reviews, see Ashwell and Harford, 1982; Breitfeld et al., 1985). Desialylation permits terminal galactose moieties to bind with high affinity to the galactose domain of ASGP-r, located at the COOH terminus and exposed to the cell surface (Chiacchia and Drickamer, 1984; Holland and Drickamer, 1985; Halberg et al., 1987). Nonglycosylated proteins and glycoproteins containing terminal mannose or N-acetylglucosamine will not bind to rat ASGP-r (Ashwell and Harford, 1982). This galactose-specific binding property of rat ASGP-r permits the purification of the receptor by ASGP-agarose (Hudgin et al., 1974) or galactose-agarose affinity chromatography (Halberg et al., 1987). ASGP-r has been isolated from rat, rabbit, and human liver in the presence of nonionic detergent (Tanabe et al., 1979; Hudgin et al., 1974; Baenziger and Maynard, 1980). Gel electrophoretic analysis has shown multiple ASGP-r species in rat and rabbit preparations (Tanabe et al., 1979; Hudgin et al., 1974). In rat liver, the major and minor forms of ASGP-r (designated rat hepatic lectin, RHL-1 and RHL-2/3, respectively) have been characterized by amino acid sequence analysis and molecular cloning (Drickamer et al., 1984; Holland et al., 1984; Halberg et al., 1987). RHL-2 and RHL-3 share a common polypeptide backbone which is distinct from RHL-1 (Halberg et al., 1987). However, RHL-2 and RHL-3 differ from each other in the extent of glycosylation (Halberg et al., 1987). Features not observed in the primary structure of RHL-1 but present in RHL-2/3 include an extra 18-amino acid segment near the cytoplasmic NH₂ terminus, a potential site for protein phosphorylation (serine residues), and the position of one of the three N-linked oligosaccharides (Drickamer et al., 1984; Halberg et al., 1987). These differences suggest that each of the rat liver ASGP-r species may perform specific functions during cell function, the nature of which awaits elucidation.

In this paper we report the purification of rat testis galactosyl receptor (RTG-r) that is antigenically related to the minor ASGP-r RHL-2/3 species of rat liver and has binding affinity for galactose. Both immunoblotting and protein-purification procedures indicate the apparent lack of a RTG-r form equivalent to the major ASGP-r RHL-1 species of rat liver. RTG-r was localized on the surface of cultured Sertoli and spermatogenic cells, spermatogenic cells extruded from seminiferous tubules, and overlying the acrosome of mature sperm.

Materials and Methods

Materials

Rats (5–100 d old) were purchased from Charles River Breeding Laboratories, Inc., Wilmington, MA. Frozen testes from sexually mature rats were from Pel-Freez Biologicals, Rogers, AR. Eagle's minimum essential medium, retinol, D-galactose, D-mannose, N-acetylglucosamine, vinyl sulfone, and Triton X-100 were from Sigma Chemical Co., St. Louis, MO. Cell

1. **Abbreviations used in this paper:** ASGP-r, asialoglycoprotein receptor; RTG-r, rat testis galactosyl receptor.

culture medium supplements transferrin, epidermal growth factor, and insulin were from Collaborative Research, Inc., Waltham, MA and testosterone from Calbiochem-Behring Corp., La Jolla, CA. Growth hormone was from Kabi AB, Stockholm, Sweden. Follicle-stimulating hormone (NIH-oFSH-S16) was provided by the National Institute of Arthritis, Diabetes and Digestive Diseases and National Hormone and Pituitary Program. Protein A-[¹²⁵I] (2–10 Ci/μg) was from New England Nuclear, Boston, MA. Molecular mass markers and Sepharose 6 B were from Pharmacia, Piscataway, NJ. Electrophoresis reagents, nitrocellulose paper, and silver staining kit were from Bio-Rad Laboratories, Richmond, CA.

Preparation of Tissue and Cell Lysates

Lysates of liver, testis, seminiferous tubules, and cells (Sertoli cells, spermatogenic cells, and sperm) were made in 10 mM Tris-Cl buffer (pH 7.8) containing 1.25 M NaCl and 1% Triton X-100 (lysis buffer). Liver, testis, and mechanically dissociated seminiferous tubules samples were sonicated in lysis buffer for 3 min and then centrifuged at 16,500 g for 5 min.

Sertoli-spermatogenic cell co-cultures were prepared from 25-d-old rats as described (Tres and Kierszenbaum, 1983; Kierszenbaum and Tres, 1988). Spermatogenic cells (mainly spermatogonia, spermatocytes, and early spermatids, identified by aceto-orcein staining) were detached 2 d after plating from Sertoli cell surfaces by shearing forces. Remaining sparse spermatogenic cells were removed by hypotonic treatment (Galdieri et al., 1981). Relatively spermatogenic cell-free Sertoli cells (as determined by phase-contrast microscopy) were left in serum-free, hormone/growth factor supplemented medium (TKM, Tres et al., 1986) for an additional 24 h and detached from the plastic substrate with lysis buffer. Cell viability was monitored by both exclusion of trypan blue dye and time-lapse image recording of spermatogenic cell-specific movements (Tres and Kierszenbaum, 1983). Cell viability was >97%.

Sperm were collected from the caudal portion of the epididymal duct by retrograde perfusion with PBS through a catheter inserted in vas deferens. Seminal fluid was centrifuged (700 g, 10 min) and the pelleted sperm washed three times in PBS before solubilization in lysis buffer.

To minimize proteolysis, tissue and cell lysate samples were placed immediately after collection in sample buffer containing SDS, plus or minus 5 mM PMSF.

Purification of Rat Liver ASGP-r and RTG-r

Receptor was purified from rat liver and testis according to the procedure originally described for the preparation of rabbit ASGP-r (Hudgin et al., 1974) except that galactose-Sepharose 6B was used as the affinity resin (Halberg et al., 1987). Galactose was conjugated to Sepharose by the method of Fornstedt and Porath (1975). A similar galactose-Sepharose affinity medium was used for the purification of rat liver RHL-1 and RHL-2/3 by Halberg et al. (1987). Mannose- and N-acetylglucosamine-Sepharose columns were also prepared and used for determining of carbohydrate-binding specificity of RTG-r.

Rat liver (4.5 g of acetone powder) and rat testis (3.2 g of acetone powder) were used to solubilize and purify the receptor on galactose-Sepharose column (1.5 × 11.5 cm). The solubilized material was loaded on the column and the column washed with several bed volumes of loading buffer (10 mM Tris-Cl [pH 7.8] containing 1.25 M NaCl, 50 mM CaCl₂, and 0.5% Triton X-100). Bound protein was eluted with 20 mM ammonium acetate (pH 5.4) containing 1.25 M NaCl and 0.5% Triton X-100 in the second bed volume of the columns (representing fractions 15–25). The flow rate was 25 ml/h and the volume of each fraction was 1.5 ml. Optical absorption was not monitored because of Triton X-100 interference. Fractions containing purified protein were analyzed by SDS-PAGE, silver-staining, and immunoblotting using affinity-purified antisera raised against rat liver ASGP-r RHL-1 and RHL-2/3.

SDS-PAGE

One-dimensional PAGE (Laemmli, 1970) was carried out after denaturation and reduction of protein samples with SDS and 2-mercaptoethanol, respectively. Purified receptor was analyzed on both 5–15% logarithmic gradient and 7.5% acrylamide gels. Proteins resolved on gels were either stained with silver nitrate or electrotransferred to nitrocellulose paper.

Immunoblotting

Protein samples resolved by SDS-PAGE (7.5%) were transferred to nitrocellulose paper (Towbin et al., 1979) and immunoreacted with antisera to

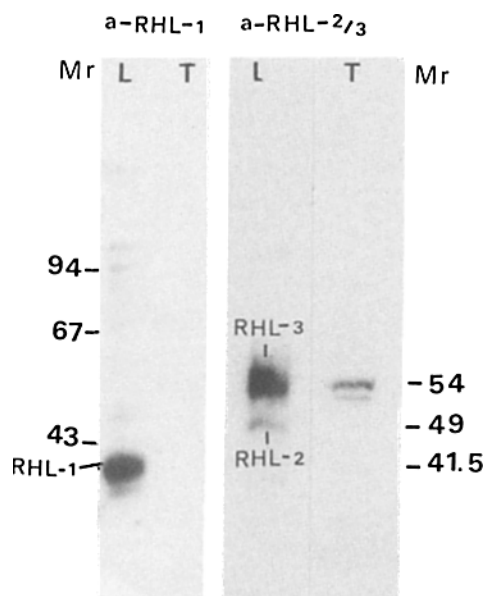


Figure 1. Immunoblot analysis of purified rat liver ASGP-r (*L*) and testis lysates (*T*) from 70-d-old rats. Protein samples (*L* = 5 μ g; *T* = 100 μ g) were resolved on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose paper, and immunoreacted with anti-RHL-1 and anti-RHL-2/3 sera (1:100 dilution). Cross-reactivity was detected with 125 I-protein A and autoradiography (24-h exposure). Numbers at the left are $M_r \times 10^3$. Numbers at the right indicate relative molecular mass of RHL-3, RHL-2, and RHL-1.

RHL-1 and RHL-2/3 (1:100 dilution in PBS). Protein A- 125 I] (1×10^5 cpm/ml) was used to detect bound antibody to receptor (Burnette, 1981). Radiolabeled protein A was localized after 24–48-h exposure of immunoblots to x-ray film using intensifying screen.

Localization of Cell-surface RTG-r by Indirect Immunofluorescence

Sertoli-spermatogenic cell co-cultures prepared on glass coverslips, epididymal sperm spread on glass coverslips, and seminiferous epithelial cells (collected on glass coverslips by squeezing gently seminiferous tubular segments from one end to the other using a small roller; Kierszenbaum et al., 1988) were fixed in 3.7% formaldehyde in PBS for 15 min. The viability of seminiferous epithelial cells collected by this procedure was monitored by placing cells on PBS followed by trypan blue-exclusion dye test and time-lapse image recording (Tres and Kierszenbaum, 1983). Cell viability was ~90%. Live, unfixed cells were immunostained in suspension according to Wolf et al. (1986). Cells were immunoreacted with anti-RHL-1 and anti-RHL-2/3 sera (1:50–1:100 dilution in PBS) for 1 h, rinsed in PBS, and treated with goat anti-rabbit IgG serum conjugated with fluorescein (1:50–1:100 dilution in PBS). Antisera were prepared in rabbits against purified RHL-1 and RHL-2/3 antigens. Antisera were affinity-purified on columns of the separated receptor species immobilized on Sepharose matrices and then rechromatographed in the presence of 2-mercaptoethanol (Holland and Drickamer, 1986; Halberg et al., 1987). The specificities of antisera were verified by immunoblotting (see Fig. 3 in Halberg et al., 1987). Control experiments included omission of first and second antisera, increasing dilutions of first antisera (1:100–1:5,000), absorption of first antisera with purified liver and testis ASGP-r fractions, and extraction of sperm with 0.9% Triton X-100 to solubilize RTG-r. All controls resulted in negative immunofluorescence (data not shown). Preparations were observed in a Leitz Ortholux II fluorescent microscope using epiillumination.

Results

Antiserum to RHL-2/3 Recognizes RTG-r

Our first approach was to purify ASGP-r RHL-1 and RHL-

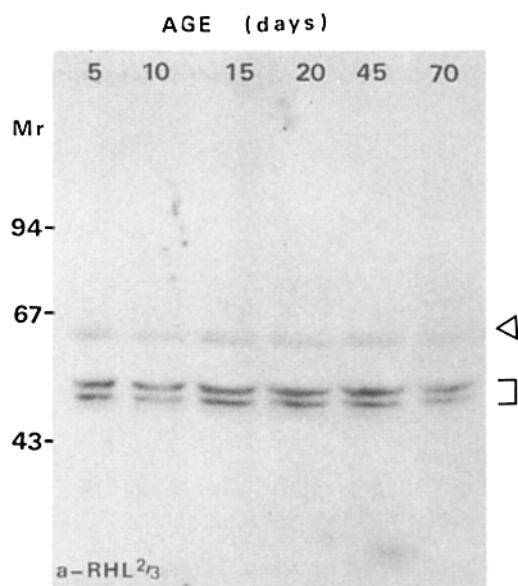


Figure 2. Immunoblot analysis of whole testis (from 5–20-d-old rats) and seminiferous tubules lysates (from 45–70-d-old rats). Samples (80 μ g/lane) were resolved on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose paper, and immunoreacted with anti-RHL-2/3 serum (1:100 dilution). Cross-reactivity was detected as described in Fig. 1. Numbers at the right are $M_r \times 10^3$. The arrowhead indicates a partially reduced or aggregated form of the receptor. The bracket identifies the RHL-3-like form of the receptor.

2/3 from rat liver and use preparations as positive controls for immunoblot analysis of testicular lysates. Our primary objective was to determine whether antisera to RHL-1 and RHL-2/3 cross-reacted with protein species in rat testis. Fig. 1 shows that anti-RHL-1 serum immunoreacted with purified rat liver RHL-1 ($M_r = 41,500$) but no immunoreactivity was detected in lysates of whole testes of sexually mature rats. However, anti-RHL-2/3 serum cross-reacted with the putative liver antigens ($M_r = 49,000$ and $54,000$, respectively) and generated an immunoreactive product in rat testis lysate in the form of a double band (M_r range = $54,000$), comigrating with RHL-3. An immunoreactive band comigrating with RHL-2 ($M_r = 49,000$) was not detected in the testis sample.

RTG-r Is Present during Testicular Development

After establishing that testes from sexually mature rats displayed a protein immunologically related to RHL-2/3 but not to RHL-1, our next approach was to determine (a) the temporal appearance of RHL-2/3-like immunoreactivity and (b) whether RHL-1-like immunoreactivity was transiently observed during testicular development. Fig. 2 depicts RHL-2/3 immunoreactive double bands (shown in Fig. 1 to comigrate with RHL-3) in testicular lysates of postnatal (days 5–10) and pubertal (days 15–20) rats, and seminiferous tubules of maturing (day 45) and sexually-mature (day 70) rats. These results indicated that RHL-2/3 immunoreactivity was present throughout testicular development and that RHL-1 immunoreactivity was absent (not shown).

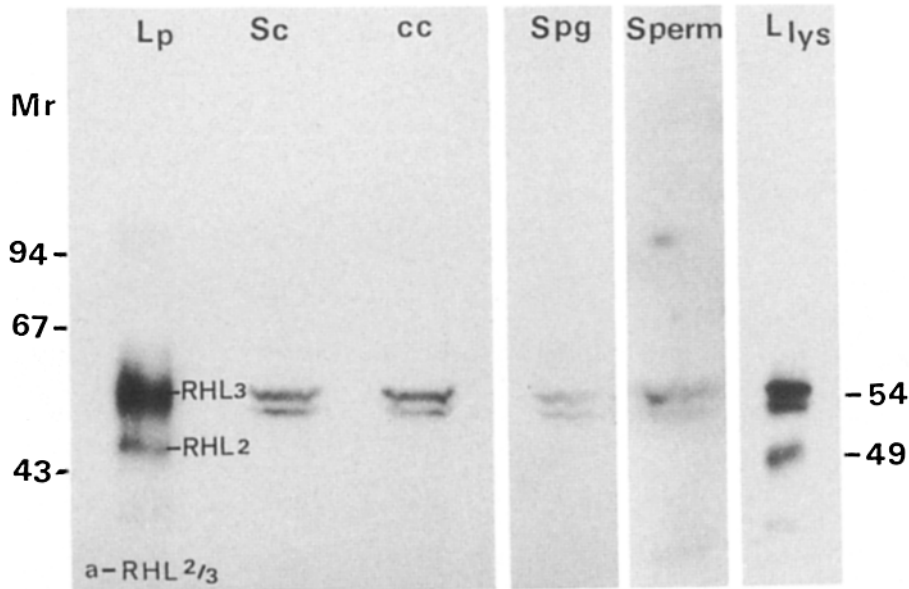


Figure 3. Immunoblot analysis of purified rat liver ASGP-r and different cell lysates using anti-RHL-2/3 serum (dilution 1:100). Samples were processed as described in Fig. 1. *Lp*, purified rat liver receptor (5 μ g); *Sc*, lysate of Sertoli cells cultured for 6 d and prepared from 25-d-old rats (200 μ g); *cc*, lysate of Sertoli-spermatogenic cells co-cultured for 5 d (200 μ g); *Spg*, lysate of spermatogenic cells detached mechanically from Sertoli cells after co-culture for 5 d (200 μ g); *Sperm*, sperm lysate, the samples collected from epididymal tail (100 μ g); *Llys*, rat liver lysate (50 μ g). Numbers at the left are $M_r \times 10^3$. Numbers at the right are relative molecular mass of RHL-3 and RHL-2.

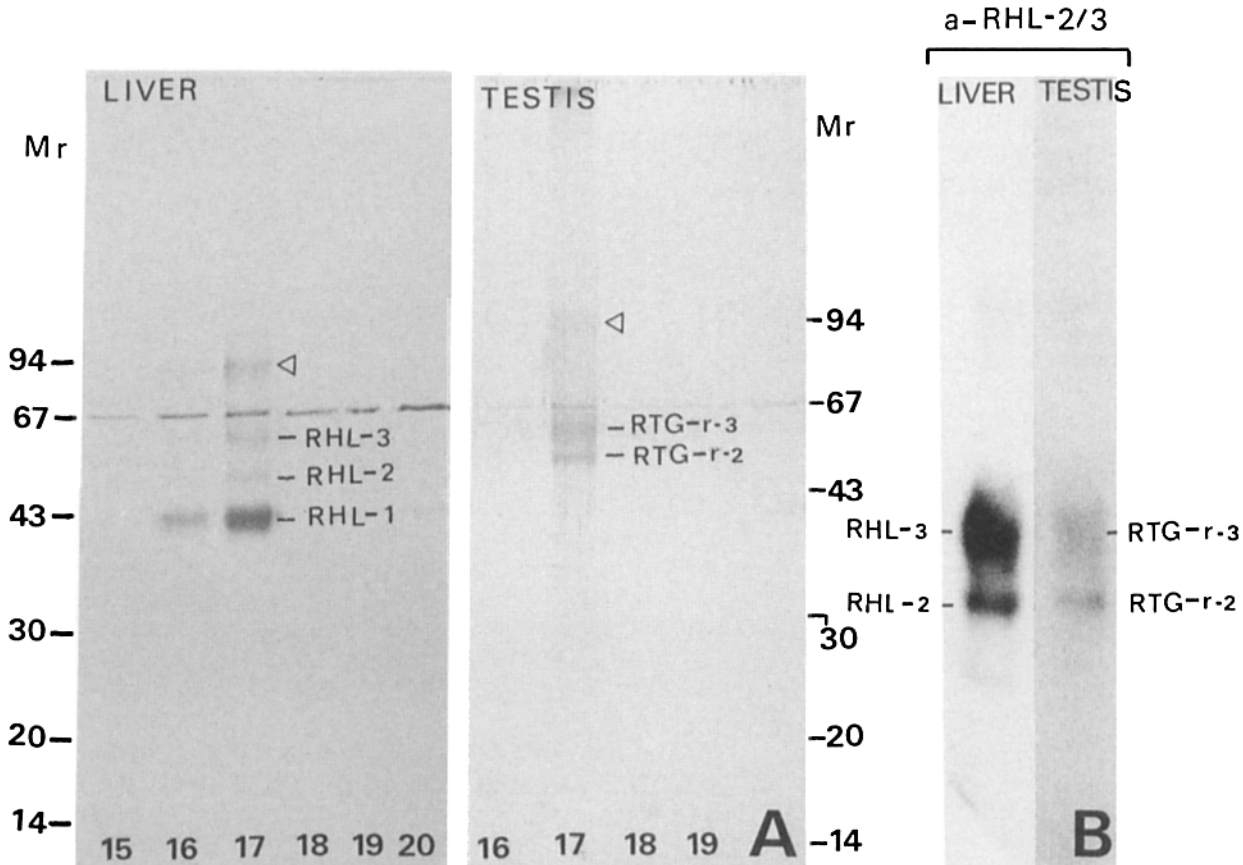


Figure 4. Purification of rat liver ASGP-r and rat testis galactosyl receptor (identified as RTG-r-3 and RTG-r-2 in *A* and *B*). (*A*) SDS-PAGE analysis (5–15% gradient polyacrylamide gel) of fractions 15–20 (indicated by numbers at the bottom) eluted from galactose-Sepharose column. Silver-stained gels. Coeluted liver RHL-3, RHL-2, and RHL-1 are observed in fraction 17. Coeluted testis RTG-r-3 and RTG-r-2 are also observed in fraction 17. The aggregated form of the receptor is indicated by an arrowhead. Numbers at right and left are $M_r \times 10^3$. The artifactual band at M_r , 67,000 is routinely detected with this silver-staining protocol. (*B*) Immunoblot analysis of purified liver and testis galactose-binding receptor. Protein samples (liver = 5 μ g; testis = 2 μ g) were resolved by SDS-PAGE (7.5% gel), transferred to nitrocellulose paper, immunoreacted with anti-RHL-2/3 serum (dilution 1:100), and processed for autoradiography. RHL-3 and RHL-2 comigrate with RTG-r-3 and RTG-r-2. Neither mannose nor *N*-acetylglucosamine coupled to agarose yielded RHL-2/3 immunoreactive polypeptides (not shown).

RTG-r Is Detected in Sertoli Cells, Spermatogenic Cells, and Sperm

To determine the cell-specific localization of RHL-2/3-like immunoreactivity, we carried out immunoblotting analyses of lysates of cultured rat Sertoli cells, Sertoli-spermatogenic cell co-cultures, detached cultured spermatogenic cells (spermatogonia, spermatocytes, and early spermatids) from co-cultures, and epididymal sperm. Fig. 3 shows the characteristic RHL-2/3-like immunoreactive double band in all samples. These results demonstrated that RHL-2/3-like immunoreactivity was associated with cultured Sertoli and spermatogenic cells and with mature sperm.

Two observations merit further comment: (a) the double-band pattern of RHL-2/3-like immunoreactivity, and (b) the apparent lack of immunoreactivity in the RHL-2 region. Fig. 3 shows that the double-band pattern is also observed in liver lysate samples (lane L_{lys}) but not in purified RHL-3 (lane L_p). Lysates collected in sample buffer (plus or minus PMSF), processed by SDS-PAGE previous repeated freezing and thawing, or processed immediately for SDS-PAGE after collection, displayed the characteristic doublet pattern. Therefore, the double-band pattern in both liver and testis lysates may be attributed to the nature of the sample and not to proteolysis.

As shown in Figs. 1-3, RHL-2-like immunoreactivity was detected in both purified and lysate liver samples but not in testicular and cell lysates (Fig. 3). Possible interpretations of this result are either a masking effect determined by an abundant cellular protein comigrating with a less abundant RHL-2-like immunoreactive protein, thus preventing immunoglobulin binding or to the partial protein electrotransfer or binding of the less abundant RHL-2-like immunoreactive protein to nitrocellulose paper. Since a polypeptide comigrating with RHL-2 was detected in galactose-Sepharose-purified testicular samples by silver staining and immunoblotting (Fig. 4), we have concluded that a technical artifact prevented the visualization of RHL-2-like immunoreactivity in testis and cell lysates.

RTG-r Has Binding Affinity for Galactose-Sepharose

Although immunoblotting studies demonstrated that rat testis, cultured Sertoli and spermatogenic cells, and mature sperm contained a protein immunologically related to the minor form rat liver ASGP-r RHL-2/3, little was known about its galactose binding activity. Fig. 4 A shows that liver samples fractionated on a galactose-Sepharose column and resolved by SDS-PAGE (5-15%) under reducing conditions yield a major fraction containing both multimeric and oligomeric RHL-1, RHL-2, and RHL-3 forms of ASGP-r. Testis samples yielded a major fraction containing two protein bands with electrophoretic mobility almost identical to RHL-2 and RHL-3, along with the multimeric form of RTG-r. Results shown in Fig. 4 A established the galactose binding affinity of RTG-r and confirmed the absence of a testis equivalent to the major rat liver ASGP-r RHL-1 form as anticipated by immunoblotting experiments (Fig. 1). Fig. 4 B demonstrates that RTG-r consists of two galactose-binding proteins which comigrate almost identically with rat liver ASGP-r RHL-2/3 and are recognized by anti-RHL-2/3 serum. Consequently, RTG-r can be regarded as both the antigenic equivalent to rat liver RHL-2/3.

Neither mannose nor *N*-acetylglucosamine conjugated to agarose yielded RHL-1 and RHL-2/3-like immunoreactive polypeptides in immunoblots (not shown). However, testicular membrane receptors for exposed mannosyl, *N*-acetylglucosaminyl, and fucosyl residues may be present but not detected by anti-RHL-1 and anti-RHL-2/3 antisera.

Redistribution of RTG-r Immunoreactive Sites during Spermatogenesis

Indirect immunofluorescence was used to determine RHL-2/3-like immunoreactive sites in Sertoli cells, spermatogenic cells, and epididymal sperm, and to confirm immunoblotting results shown in Fig. 3. Fig. 5 shows that: (a) RTG-r-immunoreactive sites on Sertoli cells surfaces have a punctuate pattern and predominate along the cytoplasmic periphery (Fig. 5 A), (b) primary spermatocytes co-cultured with Sertoli cells display a patchy, diffuse distribution (Fig. 5 B), and (c) spermatocytes detached from Sertoli cell surfaces (for immunoblotting studies, see Fig. 3, lane *Spg*) show a more uniform and diffuse distribution (Fig. 5 C). The possibility that testicular galactose-binding lectin could derive from serum proteins adsorbed to Sertoli or spermatogenic cell surfaces was regarded unlikely because the co-cultures were plated and maintained in serum-free culture medium.

Fig. 6 illustrates RHL-2/3-like immunoreactive sites in seminiferous epithelial cells collected from isolated seminiferous tubules examined by transillumination to determine specific spermatogenic stages. This approach relies on the predictable nature of the various cell associations of each stage of rat spermatogenesis (Clermont, 1972), a convenient aspect that facilitates the identification of the heterogeneous population of spermatogenic cells in the preparations by cell size and nuclear morphological criteria (Tres and Kierszenbaum, 1983). In Fig. 6, A-C, meiotic prophase spermatocytes (mainly pachytene spermatocytes) collected from spermatogenic stages I-VIII depict the same patchy diffuse pattern detected on surfaces of cultured spermatocytes (compare with Fig. 5 B). In Fig. 6, D-E, the punctuate immunoreactive product coalesces to one side of elongating nucleus and acrosome of spermatids collected from spermatogenic stages IX-XIV. An immunofluorescent phase-contrast microscopic correlation indicates that the redistribution of RTG-r on developing spermatids coincides with morphogenic acrosomal and nuclear events (data not shown). The developing spermatid tail was not immunoreactive. No apparent immunoreactivity was observed on spermatogonia. We have not determined RTG-r receptor immunoreactivity in peritubular cells or Leydig cells.

Fig. 7, A-D illustrates sperm collected from the epididymal tail immunostained with anti-RHL-2/3 serum. Immunoreactive coarse patches are restricted to dorsal surfaces of the sperm head, overlying the acrosome. We have not determined immunoreactive patterns of sperm collected from different segments of the epididymal duct. Fig. 7 E shows epididymal sperm immunoreacted with anti-RHL-1 serum. As indicated by immunoblotting studies (Fig. 1), mature sperm do not display RHL-1-like immunoreactivity. There is no evidence of immunofluorescence over sperm stained with anti-RHL-2/3 serum after sperm membrane solubilization with Triton X-100 before fixation, and in live or fixed nonpermeabilized sperm stained with absorbed anti-RHL-

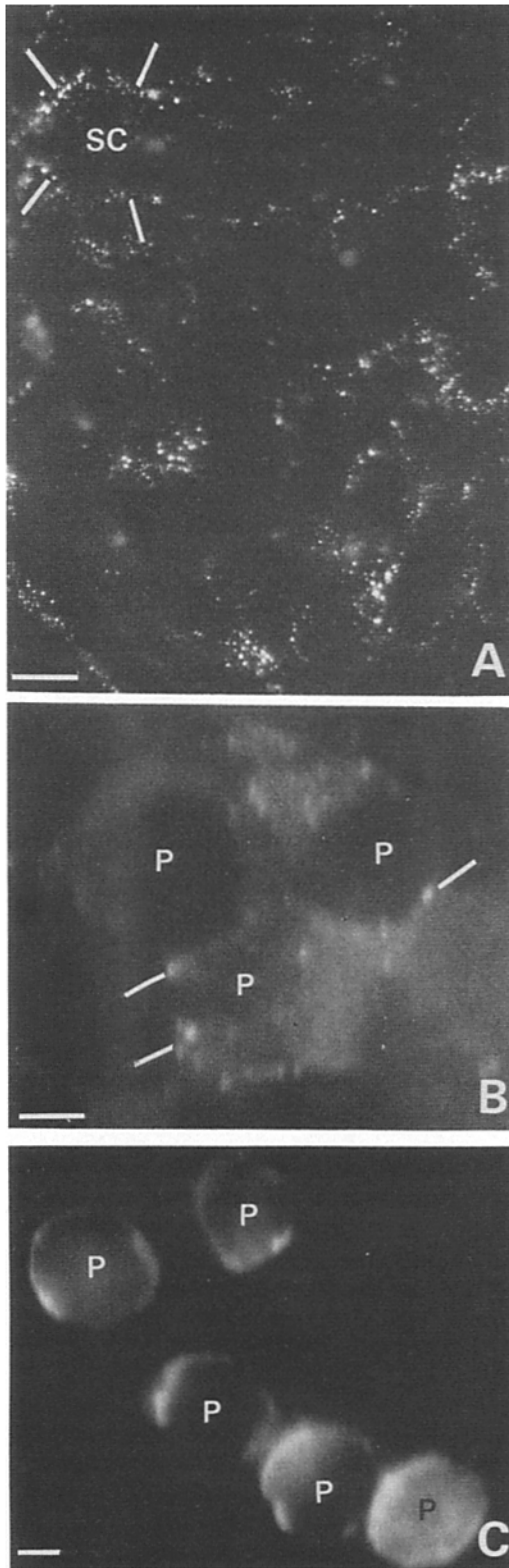


Figure 5. Indirect immunofluorescent localization of receptor immunoreactivity in cultured cells using anti-RHL-2/3 serum (dilution 1:100, second antibody 1:100). Cells illustrated in *A* and *B* were fixed in 3.7% formaldehyde in PBS for 15 min. (*A*) Cultured Sertoli cells (*SC*) with peripheral distribution of immunoreactive sites (indicated by lines). (*B*) Pachytene spermatocytes (*P*) in coculture with Sertoli cells for 6 d. (*C*) Live pachytene spermatocytes (*P*); as determined by exclusion of trypan blue dye and time-lapse image

2/3 serum with purified preparations of liver ASGP-r or RTG-r (data not shown).

Discussion

The data presented in this paper show that rat testis has a galactosyl receptor that is immunologically related to the minor polypeptide form of rat liver RHL-2/3 and has binding affinity for galactose. This galactose-binding property permits the purification to homogeneity of Triton X-100-solubilized RTG-r by one-step affinity chromatography on galactose-Sepharose. The data also show that a RTG-r polypeptide species equivalent to the major RHL-1 form is not detected in rat testis. Silver-stained gels confirmed the coexistence of RHL-1 and RHL-2/3 polypeptides after affinity chromatography of liver samples. However, only two polypeptides with electrophoretic mobility similar to RHL-2/3 were observed in testis preparations.

It is generally assumed that ASGP-r is found only on hepatocyte membranes to mediate the turnover of senescent serum glycoproteins (Ashwell and Harford, 1982). Although RTG-r and the less abundant rat liver RHL-2/3 share both antigenic homology and binding affinity for galactose, the biological role of these cell surface carbohydrate receptors is not clear. The primary structure and glycosylation heterogeneity of rat liver RHL-1 and RHL-2/3 have been reported (Holland et al., 1984; Drickamer et al., 1984; Halberg et al., 1987). It has been suggested that RHL-1 and RHL-2/3 may serve different cellular functions (Halberg et al., 1987). Consequently, since rat testis apparently lacks an equivalent to the major RHL-1 form, the testis is a valuable experimental system for determining functional differences between rat liver RHL-1 and RHL-2/3 and for establishing whether the overall structure of RTG-r correlates with that reported for RHL-2/3. This is the subject of continuing studies.

In liver, ASGP-r is localized on the sinusoidal plasma membrane domain of hepatocytes (Wall and Hubbard, 1981) where it binds glycoproteins that bear terminal galactose residues on their oligosaccharide side chains. ASGP-r/ligand complexes are internalized via coated pits and vesicles, and, after ligand uncoupling, the ASGP-r recycles to the cell surface for reuse in subsequent receptor-mediated endocytic events. Structural and kinetic aspects of the endocytosis of the receptor-ligand complex and ASGP-r recycling have been reported in liver, isolated hepatocytes, and hepatoma cells (Hubbard et al., 1979; Weigel, 1980; Zeitlin and Hubbard, 1982; Breitfeld et al., 1985). In the seminiferous tubule, Sertoli cells are specialized to perform vectorial transporting functions and maintain an intimate association with developing spermatogenic cells. RTG-r on surfaces of both Sertoli and spermatogenic cells may be potentially effective for receptor-mediated endocytosis of galactose-terminal glycoproteins from the circulation or originated in the seminiferous tubule. However, the possibility cannot be disregarded that RTG-r participates in other cell functions unrelated to receptor-mediated endocytosis. In fact, it has been reported that the simultaneous expression of cDNAs

recording) detached from Sertoli cell surfaces and immunoreacted in suspension with anti-RHL-2/3 serum. See Materials and Methods for details. Bars: (*A*) 20 μm ; (*B* and *C*) 5 μm .

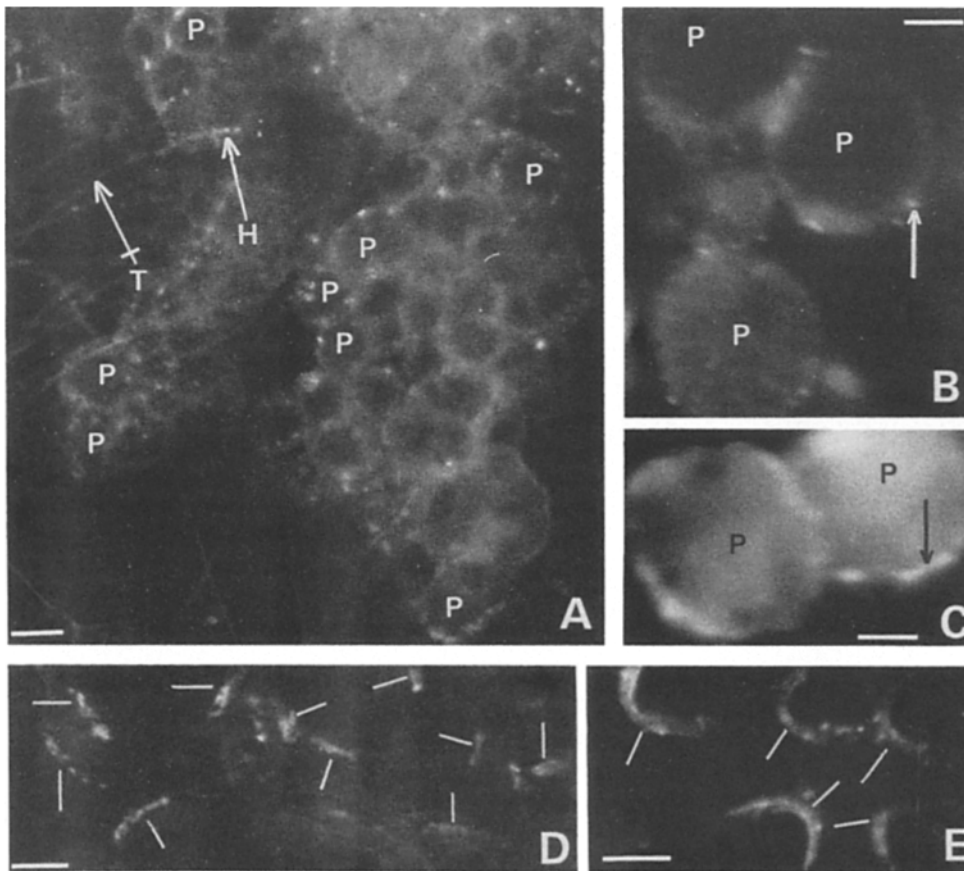


Figure 6. Indirect immunofluorescence of cells squeezed out from seminiferous tubules, fixed in 3.7% formaldehyde, and immunoreacted with anti-RHL-2/3 serum as described in Fig. 5. (A) Cluster of cells collected from seminiferous tubules stages I-VIII. Punctuate immunoreactivity is detected on the surfaces of pachytene spermatocytes (P). The arrow identifies the immunoreactive head (H) of a maturing spermatid; the crossed arrow identifies the nonimmunoreactive tail (T). (B and C) Detail of pachytene spermatocytes (P) showing punctate (arrow in B) and patchy (arrow in C) cell surface immunoreactivity. (D) Immunoreactivity overlying the elongating nucleus and developing acrosome of spermatids (indicated by lines) collected from seminiferous tubules stages IX-XIV. (E) Crescent-shaped immunoreactivity over the head of mature spermatids (indicated by lines) collected from seminiferous tubules stages VI-VIII. Bars: (A and D) 20 μm ; (B and C) 5 μm ; (E) 10 μm .

encoding RHL-1 and RHL-2/3 is required for producing a functional ASGP-r (McPhaul and Berg, 1986). These findings raise intriguing questions because only one polypeptide form antigenically and functionally related to RHL-2/3 is apparently expressed in rat testis. In addition, sperm are transcriptionally inactive (Monesi, 1965) and receptor-mediated endocytosis, if any, may not be significant. This implies that receptor-mediated endocytosis may not be the sole function of RTG-r. RTG-r is present early on during testicular development. Its early appearance suggests that it serves some biological function before the establishment of complete spermatogenesis. We are using spermatogenesis in vitro approaches (Kierszenbaum and Tres, 1987; Ueda et al., 1988) to determine a possible role of RTG-r in the interaction of Sertoli cells with developing spermatogenic cells, known to be coated by specific surface glycoproteins (Millette and Moulding, 1981; Tung and Fritz, 1978).

Immunofluorescence studies, performed under conditions that ensure detection of both cell surface and specific immunoreactivity, demonstrate (a) the temporal appearance of galactosyl receptor during meiotic prophase, (b) its localization on spermatids, in correlation with acrosomal development and nuclear condensation, and (c) its persistence on a region of the sperm plasma membrane that mediates attachment to the zona pellucida. These findings suggest that RTG-r is biologically significant early in spermatogenesis and be-

fore sperm-egg recognition. Essentially, the galactosyl receptor can be regarded as a member of sperm surface antigens appearing during spermatogenesis and retained on sperm head surfaces. During fertilization, the galactosyl receptor may recognize galactose-terminal regions of oligosaccharide chains attached to specific molecules in the zona pellucida.

An interesting parallel exists between mouse sperm surface galactosyl-transferase (GalTase, $M_r = 57,000$) and sperm surface RTG-r ($M_r = 54,000-49,000$). Both GalTase and RTG-r are present on the surface of somatic (GalTase, Pierce et al., 1980; RTG-r, this paper) and spermatogenic cells (Scully et al., 1987; this paper). During spermiogenesis, both molecules change their initial diffuse localization on the surface of meiotic prophase spermatocytes to a paracrosomal cell surface localization in maturing spermatids (Scully et al., 1987; this paper). Mouse sperm binding to egg zona pellucida may be mediated by GalTase which recognizes and binds to terminal *N*-acetylglucosamine residues in zona pellucida (Shur and Hall, 1982). In this context, mouse sperm bind to specific O-linked oligosaccharides of the sperm receptor, a glycoprotein called ZP3, present in egg zona pellucida (Bleil and Wassarman, 1980; Florman and Wassarman, 1985; for a review see Wassarman, 1987). Galactose, present in α -linkage at the nonreducing terminus of these oligosaccharides serves as an essential binding de-

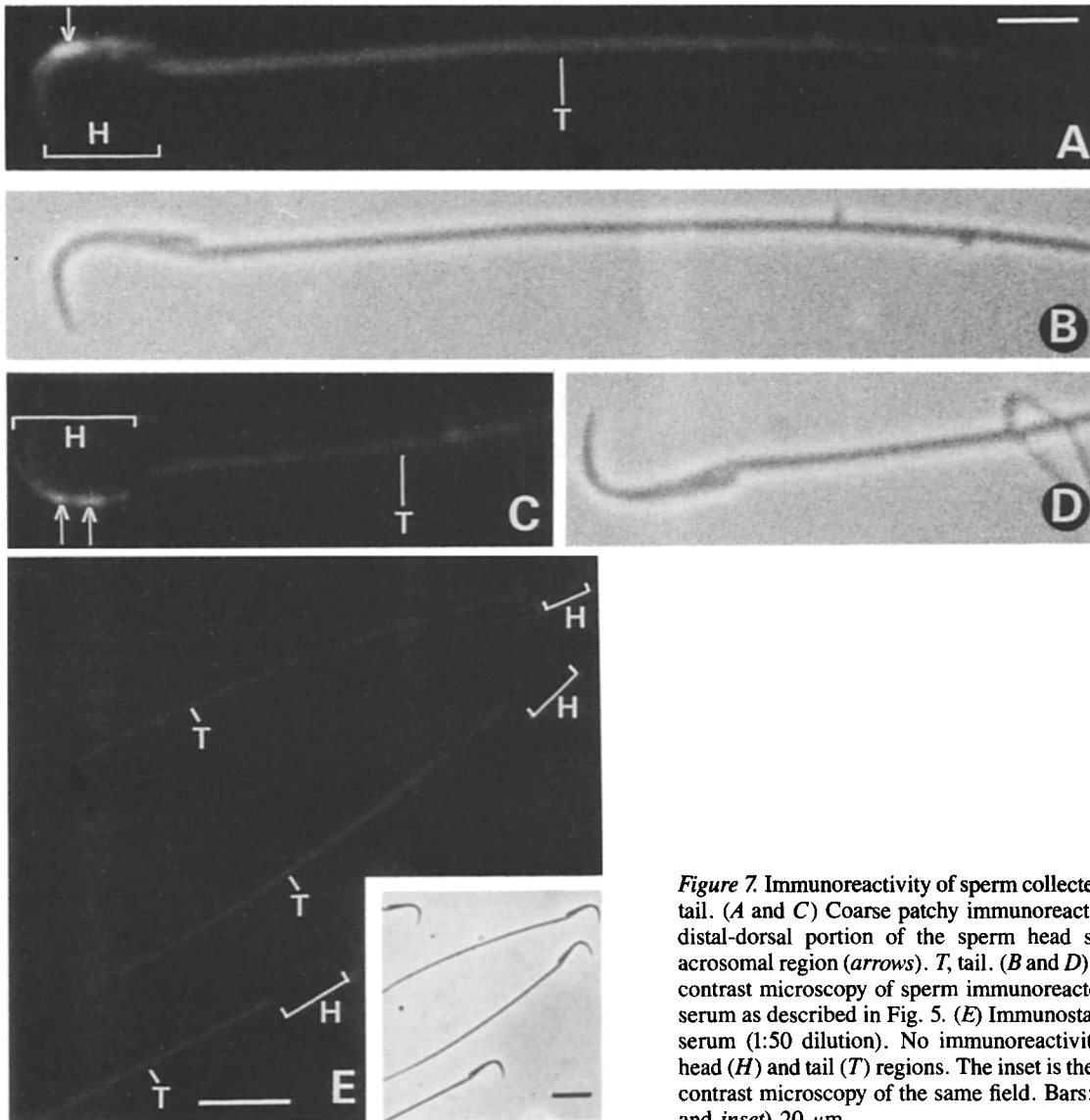


Figure 7. Immunoreactivity of sperm collected from the epididymal tail. (A and C) Coarse patchy immunoreactivity detected over the distal-dorsal portion of the sperm head surface, overlying the acrosomal region (arrows). T, tail. (B and D) Corresponding phase-contrast microscopy of sperm immunoreacted with anti-RHL-2/3 serum as described in Fig. 5. (E) Immunostained with anti-RHL-1 serum (1:50 dilution). No immunoreactivity can be detected on head (H) and tail (T) regions. The inset is the corresponding phase-contrast microscopy of the same field. Bars: (A and B) 10 μm ; (E and inset) 20 μm .

terminant on the sperm receptor (Bleil and Wassarman, 1988). In fact, RTG-r binds to galactose coupled to agarose. A more detailed understanding of the structure and function of RTG-r is required before ascribing roles in spermatogenesis and fertilization.

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