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Late-Stage Spermatids Are Characterized by Expression of the "Liver-Specific" Asialoglycoprotein Receptor, RHL-1

BRIAN E. HUBER

Division of Experimental Therapy, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

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SUMMARY

The major and minor forms of the asialoglycoprotein receptor (ASGP-R), designated in the rat as RHL-1 and RHL-2/3, respectively, have traditionally been considered to be expressed exclusively in hepatic parenchymal cells. Northern blot analysis now demonstrates that rat and mouse testis express a receptor similar to the RHL-1 ASGP-R but not the RHL-2/3 receptor. *In situ* hybridization studies demonstrate that late-stage mouse and rat spermatids are the testicular cells that express the RHL-1 ASGP-R. The rat spermatid RHL-1 receptor has functional binding capability, with a K_D of approximately 1.4×10^{-8} M, and similar characteristics, compared with the hepatic RHL-1 receptor, regarding ligand binding specificity and ion dependence. These findings have major implications for therapeutic procedures attempting to target cytotoxic agents or DNA to hepatocytes using the ASGP-R. In addition, these findings demonstrate that late-stage spermatids are transcriptionally active and suggest that the RHL-1 receptor may have a functional role in sperm maturation and/or fertilization.

The hepatic ASGP-R binds and internalizes specific serum glycoproteins that are desialylated, exposing galactose or *N*-acetyl-galactosamine residues (for review, see Refs. 1–3). The processes of ligand endocytosis, intracellular processing, and receptor cycling have been extensively studied (as examples, see Refs. 4–7). The rat ASGP-R consists of two species, a major form (RHL-1) and a minor form (RHL-2/3) (8–12). Amino acid sequence analysis has demonstrated that RHL-1 and RHL-2/3 are very homologous, except that RHL-2/3 contains an 18-amino acid insertion near the amino terminus (11, 12). RHL-2 and RHL-3 are identical regarding their polypeptide backbone but differ in post-translational carbohydrate modifications (10–12). The physiological significance of the difference between RHL-1 and RHL-2/3 in normal cell function has not yet been determined, but recent studies suggest that both forms of the receptor may be essential to form a functional receptor (13, 14).

Traditionally, the ASGP-R is believed to be expressed exclusively in parenchymal hepatocytes. As such, the utility of the ASGP-R to function as a hepatocyte-targeting receptor has been explored. For example, adenine arabinoside 5-monophosphate was coupled to galactosyl-terminating glycoproteins to target adenine arabinoside 5-monophosphate to hepatocytes chronically infected with hepatitis virus (15, 16), a double-stranded DNA plasmid complexed to asialoorosmucoid/poly-L-lysine was constructed to target a foreign gene to hepatocytes (17), and ASF-tacked liposomes containing interferon-γ were used to target hepatoma cells containing hepatitis B virus (18). These findings suggest a rational approach for selective drug and gene delivery to the hepatocyte. However, the rationale for these procedures is based on the assumption that the ASGP-R is expressed exclusively in the hepatocyte. This may not be the case. Recent evidence suggests that peritoneal macrophages express a galactose/*N*-acetylgalactosamine-specific binding lectin that has homology to RHL-1 (19). Immunohistochemical studies have also indicated that the minor form of the rat ASGP-R, RHL-2/3, may also be found on Sertolic cells, spermatogenic cells, and epididymol sperm (20). We now report that late-stage mouse and rat spermatids express a functional RHL-1 receptor. These findings may have significant implications for therapeutic approaches that attempt to deliver either cytotoxic compounds or genes to the hepatocyte via the ASGP-R. In addition, these findings may provide insight into the normal processes of spermatogenesis and/or fertilization.

Experimental Procedures

Materials. The molecular hybridization probes were as follows: the RHL-1 probe was a 1169-bp cDNA probe designated clone 22 (11), and the RHL-2/3 probe was an approximately 700-bp cDNA probe designated clone 7 (12). These probes were supplied by K. Drickamer (Columbia University, New York). Although RHL-1 and RHL-2/3 have significant homology, these probes extend significantly into the 3′ untranslated region of the cDNAs for both genes, a region that is quite divergent between the two genes. An actin cDNA probe was used as a hybridization control. ASF and fetuin were obtained from Sigma Chem-

ABBREVIATIONS: ASGP-R, asialoglycoprotein receptor; SSC, standard saline citrate; ASF, asialofetuin; bp, base pairs; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

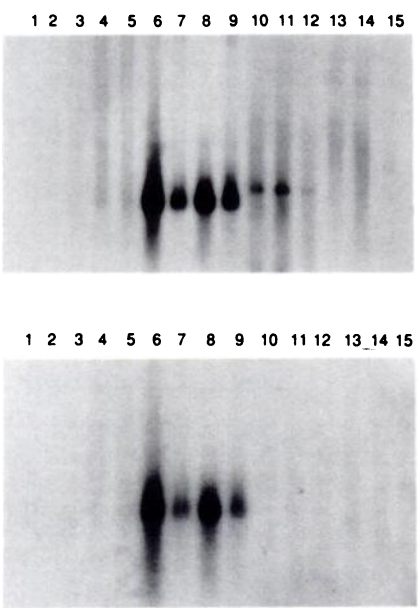

Fig. 1. Steady state levels of RHL-1-associated and RHL-2/3-associated transcripts in different rat organs. Poly(A)$^+$ RNA was isolated from Morris rat hepatoma 7777 cells (*lanes 1* and *2*), rat brain (*lane 3*), rat spinal cord (*lane 4*), rat lung (*lane 5*), rat liver (*lanes 6–9*), rat testis (*lanes 10* and *11*), rat spleen (*lane 12*), rat kidney (*lane 13*), rat small intestine (*lane 14*), and rat gluteus maximus striated muscle (*lane 15*). All lanes contain 5 μg of poly(A)$^+$ RNA, except *lanes 6* and *8*, which contain 10-μg samples. Morris rat hepatoma cells act as a negative control poly(A)$^+$ RNA sample (see Ref. 18). RHL-1-associated (A) and RHL-2/3-associated (B) transcripts were detected by hybridization and autoradiography, as described in Experimental Procedures. Rehybridization of this blot with an actin cDNA probe confirmed that each lane contained approximately equal quantities of RNA, except that *lane 15* appeared to have a slightly greater amount of actin transcripts.

ical Co. and GIBCO/BRL, respectively, and were further purified using an Amicon PM 30 membrane.

Northern blot analysis. Poly(A)$^+$ RNA was isolated and Northern blot analysis was performed by methods previously described (21, 22). To distinguish between RHL-1- and RHL-2/3-specific transcripts, blots were washed under stringent conditions (0.1 × SSC/0.1% sodium dodecyl sulfate, 46°, 60 min), in which the probes do not cross-hybridize.

***In situ* hybridization.** *In situ* hybridization was performed using ^{35}S-riboprobes. A *Pst*I/*Pst*I fragment and an *Eco*RI/*Eco*RI fragment were isolated from clone 22 and clone 7, respectively. These fragments were inserted into the multiple cloning site of pBluescript II KS(+) (Stratagene Corp.), with subsequent generation of sense and antisense RNA transcripts using either T7 or T3 RNA polymerase (20 units), in a 20-μl reaction mixture containing 40 mM Tris·HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.5 mM ATP, CTP, and GTP, 20 μM UTP, and 3.7 μM ^{35}S-UTP (1300 Ci/mmol). Template DNA was removed using RNase-free DNase (RQ1; Promega BioTeck).

Adult rat liver and adult rat and mouse testes were fixed at 4°, in phosphate-buffered saline containing 4% paraformaldehyde, for 2 hr. The fixative was then made 0.1% in sodium deoxycholate and 0.1% in Triton X-100, and fixation continued for an additional 15 hr. Tissues were then embedded in paraffin and sectioned at a thickness of 7 μm, and sections were attached to slides precoated with poly-L-lysine. Paraffin was removed with xylene treatments, and the sections were rehydrated. The sections were pretreated for 5 min in 0.2 N HCl, digested for 5 min in proteinase K (1 μg/ml), and acetylated for 5 min in 0.1 M triethanolamine containing 0.25% acetic anhydride. Prehybridization was done for 2 hr at 55° in 50% deionized formamide, 0.6 M NaCl, 10 mM Tris·HCl (pH 7.5), 1 mM EDTA, 10 mM dithiothreitol, 0.5 mg/ml sheared genomic DNA, 0.5 mg/ml yeast tRNA, 10% dextran sulfate, 1× Denhardt's solution (1× Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin). Hybridization with either sense or antisense riboprobes was performed for 18 hr at 55°, in the prehybridization solution. The slides were then washed at 55° for 30 min in 2× SSC (1× SSC = 0.15 M NaCl, 0.015 M trisodium citrate), incubated at 37° for 30 min in 2× SSC containing DNase-free RNase (50 μg/ml), and then washed for 60 min at 55° in 0.1× SSC containing 14 mM 2-mercaptoethanol and 0.1% tetrasodium pyrophosphate. Dried slides were dipped in Kodak NTB-2 autoradiography emulsion and exposed for 15 days. After development of the photographic signal, the tissue was counterstained with hematoxylin and eosin or methyl green.

ASF surface binding in isolated epididymal sperm. Specific binding of ^{125}I-ASF and ^{125}I-fetuin to epididymal sperm was determined. Rat epididymis were removed, and the sperm exudates were washed at 37° in phosphate-buffered saline containing no $CaCl_2$. The sperm were then placed in binding buffer (Dulbecco's modified Eagle's medium/F12, supplemented with $CaCl_2$ to a final concentration of 5 mM) at 4°, washed three times, and suspended at a final density of 4×10^6 cells/ml. Total surface binding of ^{125}I-ASF or ^{125}I-fetuin was done at 4° for 60 min, by methods previously described (21, 23, 24). Nonspecific binding was determined using a 600-fold excess of unlabeled ASF or unlabeled fetuin. Specific binding was determined by subtracting nonspecific binding from total binding. Receptor internalization was assessed by the methods of Bridges *et al.* (23).

***In vivo* studies.** ^{125}I-ASF (2.6×10^8 dpm/injection; 8.87×10^6 dpm/μg of ASF) and [^{3}H]inulin (4×10^7 dpm/injection; 1 Ci/mmol) were coinjected, in 0.3 ml of saline, into the tail vein of male rats. At 3, 6, 12, 16, and 45 min after injection, the animals were killed and the liver, testes (plus epididymis), spleen, lung, and gluteus maximus striated muscle were quickly removed, washed in phosphate-buffered saline, and placed into liquid nitrogen. The tissues were homogenized (5 volume equivalents), and radioactivity was determined in 20-mg samples by either γ emission or β emission, using a Packard model 1900 scintillation counter with dual quench curves and an ^{125}I/^{3}H dual counting program. Inulin was used to determine the percentage of the homogenate contributed by blood, to correct for the differences in blood content and perfusion in the different tissues.

Results

Northern blot analysis. To determine whether RHL-1 and/or RHL-2/3 are transcribed in nonhepatic tissues, Northern blot analysis was performed. Poly(A)$^+$ RNA was isolated from rat liver, kidney, spleen, brain, spinal cord, testis, lung, small intestine, and gluteus maximus striated muscle and was hybridized to RHL-1- or RHL-2/3-specific probes under stringent conditions. There was specific hybridization to liver and testis poly(A)$^+$ RNA samples using the RHL-1 probe (Fig. 1). There also appeared a very weak hybridization signal to spleen poly(A)$^+$ RNA. RHL-1 transcripts in the liver were approximately 1.4 kilobase, which is consistent with previous reports (11, 21). However, the RHL-1-related transcripts in the testis were approximately 1.5 kilobase, and the steady state level was approximately one tenth the steady state level observed in the liver. When this blot was rehybridized to the RHL-2/3-specific probe, only RHL-2/3-specific transcripts were detected in liver poly(A)$^+$ RNA samples. Poly(A)$^+$ RNA was then isolated from mouse testis and hybridized to the RHL-1- and RHL-2/3-specific probes. Similar to rat testis, there was specific hybridization to mouse testis poly(A)$^+$ RNA using the RHL-1-specific probe but not the RHL-2/3-specific probe. These data indicate that rat and mouse testis, and possibly rat spleen, transcribe

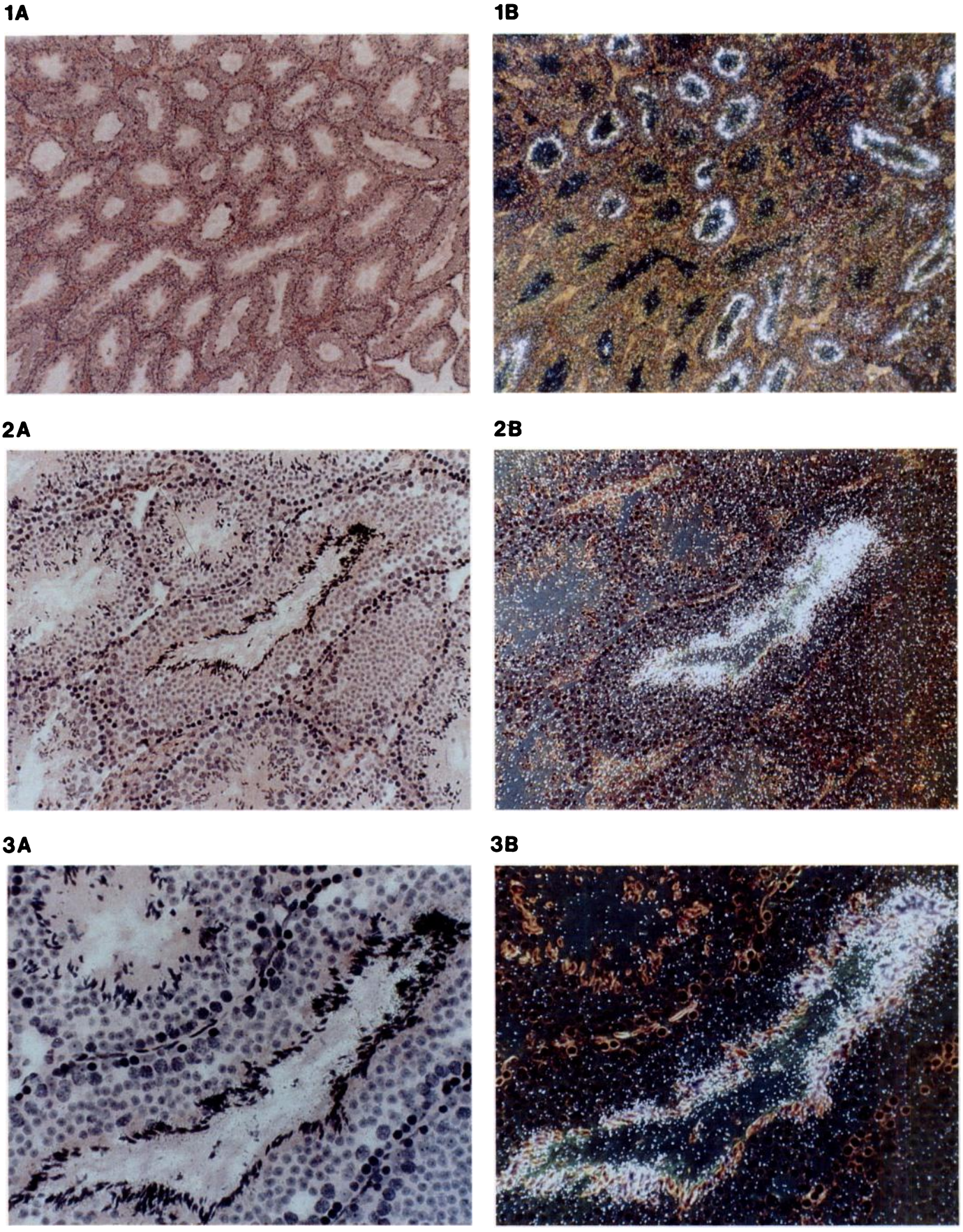

Fig. 2. *In situ* hybridization of RHL-1 antisense riboprobes in the testis. *In situ* hybridization was performed using ^{35}S-labeled sense and antisense riboprobes for the RHL-1 and RHL-2/3 ASGP-R. Tissue fixation and hybridization were performed as described in Experimental Procedures. Represented are low (*1*), moderate (*2*), and high (*3*) magnifications of mouse seminiferous tubules hybridized to the antisense RHL-1 riboprobe and photographed under bright field (A) or dark field (B). No specific hybridization was detected using the sense RHL-1, antisense RHL-2/3, or sense RHL-2/3 riboprobes.

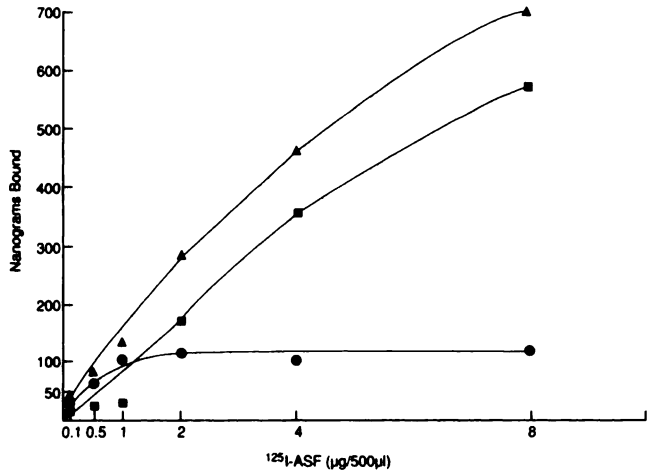

Fig. 3. Specific binding of ASF to rat epididymal sperm. Total and nonspecific binding of ^{125}I-ASF to rat epididymal sperm was determined as described in Experimental Procedures. Specific binding was determined by subtracting nonspecific binding from total binding. Each *point* represents the average of duplicate or triplicate determinations. ▲, Total binding; ■, nonspecific binding; ●, specific binding.

TABLE 1

***In vivo* distribution of radioactivity**

^{125}I-ASF (2.6×10^6 dpm) and [^{3}H]inulin (4×10^7 dpm) were coinjected in 0.3 ml of saline, into the tail vein of male rats. At 3, 6, 12, 16, and 45 min after injection, the animals were killed, the liver, testes (plus epididymis), spleen, lung, and gluteus maximus striated muscle were removed and homogenized (5 volume equivalents), and radioactivity was determined in 20-mg samples, by either γ emission or β emission. Inulin was used to determine the percentage of the homogenate contributed by blood, to correct for the differences in blood content and perfusion in the different tissues. Data for the 3-min time point are given. No increase in ASF uptake in the testicular plus epididymal compartment was seen up to 45 min. Data are expressed as percentage of counts in the liver compartment.

Organ	Uptake	
	^{125}I β emission with ^{3}H correction[a]	^{125}I γ emission
	%	
Liver	100.0	100.0
Testis plus epididymis	0.4	0.5
Spleen	7.0	7.2
Lung	3.2	3.2
Gluteus maximus	0.5	0.7

[a] [^{3}H]Inulin was used to calculate the percentage of the homogenate contributed by the blood. This correction factor was used to calculate the ^{125}I β emissions contributed by the tissue minus the blood content.

an RHL-1-associated gene. No RHL-2/3-associated gene transcripts could be detected within the limits of Northern blot analysis.

***In situ* hybridization.** The testis is a complex organ composed of multiple cell types. To identify the cell type in the testis expressing the RHL-1-associated transcript, *in situ* hybridization studies were performed using sense and antisense ^{35}S-riboprobes for RHL-1 and RHL-2/3. Antisense RHL-1 riboprobes produced specific and intense hybridization restricted to late-stage mouse and rat spermatids (Fig. 2). Hybridization appeared to be restricted to stage VIII spermatids at the seminiferous epithelial cell surface and in the lumen of the seminiferous tubule. No hybridization was observed in type A, intermediate, or type B spermatogonia, spermatocytes, Sertoli cells, Leydig cells, or residual bodies, using the RHL-1 antisense probe. The specific hybridization signal obtained using the RHL-1 antisense riboprobe in late-stage spermatids could be eliminated if the sections were preincubated with RNase. No specific hybridization was observed using the sense RHL-1 riboprobe, antisense RHL-2/3 riboprobe, or sense RHL-2/3 riboprobe. These data indicate that late-stage spermatids actively transcribe an RHL-1-associated gene.

ASF surface binding in isolated epididymal sperm. To ascertain whether the spermatic RHL-1 ASGP-R is functional, rat epididymal sperm were isolated, and high affinity, specific, cell surface binding of ^{125}I-ASF and ^{125}I-fetuin was determined. Fig. 3 illustrates that rat epididymal sperm exhibited specific, high affinity binding to ASF, with a K_D of approximately 1.4×10^{-8} M (assuming that the molecular weight of ASF is 44,000 and 1 mol of receptors binds 1 mol of ASF). Specific cell surface binding was reduced by 65% in the presence of 25 mM EDTA. No specific binding was detected using 0.5, 1.0, and 2.0 μg of ^{125}I-fetuin as the binding ligand.

Internalization of the sperm RHL-1 receptor was analyzed by methods previously described (23). However, once the radiolabeled ligand was bound to sperm at 4° in the absence of EGTA or EDTA, little of this radioactivity could then be displaced using the methods of Bridges *et al.* (23). This discrepancy precluded the utilization of a standard endocytosis assay for the ASGP-R, and alternative methodologies must be used to address this question.

***In vivo* studies.** Rats received coinjections, in the tail vein, of ^{125}I-ASF and [^{3}H]inulin. At various times, liver, testis, lung, spleen, and gluteus maximus striated muscle were removed and radioactivity was determined, to assess specific organ accumulation of blood-borne ASF. Significant ^{125}I radioactivity was found in the liver just 3 min after injection (Table 1). However, there was no significant accumulation of ^{125}I radioactivity in the other organs, including the testis. The lack of radioactivity in the testicular compartment may result from the inhibition of transport of ASF through the blood-testis barrier. These data suggest that the physiological function of the sperm RHL-1 receptor may be quite distinct from that of the hepatic ASGP-R.

Discussion

We have shown that an RHL-1 ASGP-R-associated gene is transcribed in late-stage mouse and rat spermatids. The rat spermatid RHL-1-associated transcript codes for a functional receptor exhibiting properties similar to those of the hepatic RHL-1 ASGP-R. The sperm receptor has high affinity binding sites for ASF, but not fetuin, and is ion dependent. However, the size of the spermatid RHL-1 transcript is slightly larger than that of the hepatic RHL-1 transcript. This may be the result of differences in primary structure of the gene, use of alternative start or polyadenylation signals, or alterations in splicing. It is interesting to note that the human H2 ASGP-R has two associated transcripts, resulting from the alternate splicing of a 15-bp miniexon (25). In addition, it has been demonstrated that spermatogenic cells may have transcripts that differ in size from those of somatic cells. Germ cell-specific transcripts have been reported for cAMP-dependent protein kinase, RNA helicase, proenkephalin, proopiomelanocortin, cholecystokinin, and several protooncogenes (for review, see Ref. 26). The RHL-1 receptor may be another gene with germ cell-specific transcripts.

These results, showing that mature rat and mouse spermatids express a functional RHL-1 ASGP-R, are contrary to the findings of Abdullah and Kierszenbaum (20), who found only RHL-2/3 expression, based on immunodetection. The reason for this discrepancy is presently unknown. One can speculate that the receptor found in sperm has significant homology to the liver RHL-1 receptor at the nucleic acid level but is lacking the antigenic epitope recognized by the RHL-1 antibody used by Abdullah and Kierszenbaum (20).

The discovery that mature spermatids express a functional RHL-1 ASGP-R has two significant implications. First, it has been proposed that the ASGP-R can be utilized as a specific hepatocyte delivery system for cytotoxic agents or exogenous DNA. This targeting procedure could increase efficacy and decrease inadvertent and dose-limiting systemic toxicity of cytotoxic agents used in the treatment of hepatitis virus infection and primary or metastatic cancer in the liver (15, 16, 18). Therapeutic approaches using the ASGP-R to target cytotoxic agents and exogenous DNA to hepatocytes may be significantly affected by the finding that germ cells also express a functional ASGP-R. Cytotoxic agents, especially those that interact with nucleic acids, can cause sterility, heritable mutations, or congenital malformations if inadvertently concentrated in sperm.

The second significant implication of mature spermatids expressing a functional ASGP-R concerns the function of the spermatic RHL-1 ASGP-R and normal spermatogenesis. It does not appear that this receptor has the same functional role as the hepatic ASGP-R. Serum asialoglycoproteins, which are efficiently removed by the hepatic receptor, are not concentrated in the testicular or epididymal compartment by the spermatic receptor. This difference may result from the blood/testis barrier or other differences in perfusion and permeability of the testis. It is then likely that the spermatic RHL-1 ASGP-R has a role intrinsic to spermatogenesis or fertilization. Spermatozoa released from the testis are essentially infertile. Functional maturity, including alterations in metabolism, motility, and ability to bind to the zona pellucida, is achieved when the spermatozoa are exposed to the luminal environment of the epididymis (for review, see Ref. 27). The processes of sperm maturation are not well understood and represent central questions in reproductive physiology. It has been demonstrated, however, that the epididymis releases glycoproteins containing galactose or *N*-acetyl-D-galactosamine residues (28–30). Some of these glycoproteins have been shown to bind specifically to epididymal sperm but not immature testicular spermatids (28–30). Interference with epididymal binding of these glycoproteins eliminates the capacity of the sperm to fertilize an ovum (31). It is likely that specific binding of certain essential epididymal glycoproteins to the spermatozoa is the result of expression of the ASGP-R in the late-stage spermatids at the time of release from the seminiferous tubule. Because the ASGP-R is expressed at the time of release from the seminiferous tubule, retrograde epididymal secretions may be able to modify the fertilizing capacity of the spermatozoa without the necessity for epididymal passage (32).

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Send reprint requests to: Brian E. Huber, Wellcome Research Laboratories, 3030 Cornwallis Road, Research Triangle Park, NC 27709.