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Role of Endocytosis in Cellular Uptake of Sex Steroids

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Summary

Androgens and estrogens are transported bound to the sex hormone binding globulin (SHBG). SHBG is believed to keep sex steroids inactive and to control the amount of free hormones that enter cells by passive diffusion. Contrary to the free hormone hypothesis, we demonstrate that megalin, an endocytic receptor in reproductive tissues, acts as a pathway for cellular uptake of biologically active androgens and estrogens bound to SHBG. In line with this function, lack of receptor expression in *megalin* knockout mice results in impaired descent of the testes into the scrotum in males and blockade of vagina opening in females. Both processes are critically dependent on sex-steroid signaling, and similar defects are seen in animals treated with androgen- or estrogen-receptor antagonists. Thus, our findings uncover the existence of endocytic pathways for protein bound androgens and estrogens and their crucial role in development of the reproductive organs.

Introduction

Steroid hormones are lipids with important regulatory functions. They exert main regulatory activities by entering target cells and associating with nuclear hormone receptors that, in turn, act as transcriptional activators of steroid-responsive genes. Rather than being transported by lipoproteins like cholesterol, these cholesterol derivatives are bound by specific carrier proteins in the extracellular space. Such carriers include the sex hormone binding globulin (SHBG) and the corticosteroid binding globulin (CBG), transporters for sex steroids and corticosteroids, respectively (Hammond and Bocchinfuso, 1995; Scrocchi et al., 1993). According to the free hormone hypothesis, only free steroids are biologically relevant, whereas carrier bound steroids are inactive because they are blocked from entering target cells (Mendel, 1989).

Similar to steroid hormones, vitamins A and D are also transported by plasma carriers. Previously, these lipophilic metabolites were also believed to solely enter cells by free diffusion. However, we recently demonstrated the existence of endocytic pathways for tissue-specific uptake of the complexed vitamins. Megalin is a member of the LDL receptor gene family and the first confirmed endocytic receptor for carrier bound vitamins A and D. The receptor is expressed in epithelial cells of the renal proximal tubules where it binds complexes of 25-OH vitamin D_3 with the vitamin D binding protein (DBP) and vitamin A with the retinol binding protein (RBP). Following internalization, the carriers are degraded in lysosomes while the lipids are released into the cytosol for further metabolism (Christensen et al., 1999; Nykjaer et al., 1999). Endocytosis of vitamin/carrier complexes in the proximal tubules prevents urinary loss of vitamins filtered through the glomerulus, and it delivers the precursor 25-OH vitamin D_3 to tubular cells for conversion into 1,25-$(OH)_2$ vitamin D_3, a potent regulator of calcium homeostasis. Loss of megalin expression in knockout mice results in vitamin D deficiency and in bone-calcification defects, underscoring the importance of this endocytic pathway for vitamin D action in vivo (Nykjaer et al., 1999). Today, the significance of endocytosis of lipid/carrier complexes for tissue-specific delivery of vitamins A and D is well appreciated. However, this mode of lipid uptake is considered unique to the renal vitamin metabolism and perhaps not relevant for uptake of other carrier bound lipids such as steroid hormones.

Besides in the renal proximal tubules, megalin is expressed in a number of steroid-responsive tissues, in particular in the male and female reproductive organs (epididymis, prostate, ovaries, uterus) (Zheng et al., 1994). This observation raises the intriguing possibility that the role of megalin may not be restricted to endocytosis of vitamins but may extend to the cellular uptake of steroid hormones such as androgens and estrogens. Previous experimental evidence suggested the existence of cell-surface binding sites for SHBG in steroid-responsive tissues including uterus, epididymis, and prostate. Their identity and their significance for sex-steroid activity remained controversial (Krupenko et al., 1994; Rosner et al., 1999; Turner and Roddy, 1990).

Here we investigated the possible role of megalin in the cellular delivery of sex steroids and the consequences of receptor deficiency for androgen and estro-

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gen action in vivo. We demonstrate that, in cultured cells, megalin internalizes complexes of sex steroids bound to SHBG. Following internalization, the carrier is degraded in lysosomes while the steroids are released to induce steroid-responsive genes. Lack of receptor expression in knockout mice results in testicular maldescent and vaginal obstruction, two phenotypes consistent with insensitivity of megalin-deficient tissues to androgen and estrogen signals.

Results

Androgen and estrogen binding proteins are present in most body fluids, the most common being SHBG, a homodimeric protein produced in the liver and secreted into the circulation in humans (Hammond and Bocchinfuso, 1995). In rodents, this protein is known as the androgen binding protein (ABP) that is mainly produced by the fetal liver (Sullivan et al., 1991) and by Sertoli cells of the adult testes (Feldman et al., 1981). Because megalin is expressed in sex-steroid target tissues, we tested involvement of this receptor in the cellular uptake of steroid/SHBG complexes.

To do so, we developed a eukaryotic expression system to produce recombinant SHBG that closely resembles the native human protein in terms of steroid binding activity and ability to bind to membranes from steroidogenic tissues (Hilpert et al., 2001). Recombinant SHBG bound to purified megalin in vitro, as shown by surface plasmon resonance analysis (Figure 1A). The affinity of binding (K_d = 200 nM) was similar to that of DBP to megalin (Nykjaer et al., 1999). No SHBG binding was seen when the receptor was reduced, documenting specificity of the interaction (Figure 1A).

Brown Norway rat choriocarcinoma (BN16) cells express megalin but no related endocytic receptors (see Figure S1A in the Supplemental Data available with this article online). They are commonly used as a model system to evaluate cellular megalin activity. When SHBG was labeled with iodine and added to BN16 cells, efficient uptake and degradation of the protein was observed (Figure 1B, filled columns). Cellular catabolism of [^{125}I]SHBG was blocked by the addition of the receptor-associated protein (RAP), an antagonist of ligand binding to megalin (Nykjaer et al., 1999) (Figure 1B), and by leupeptin and pepstatin, inhibitors of lysosomal proteases (data not shown). Megalin-mediated uptake of SHBG was not affected by the addition of testosterone to the culture medium (Figure 1B, open columns). RAP-inhibitable uptake and degradation was also seen for ^{125}I-labeled rat and murine ABP (Figure 1B, striped and hatched columns, respectively).

To explore the effect of this endocytic pathway for SHBG/ABP on the cellular metabolism of testosterone, we determined uptake of [^{3}H]testosterone in BN16 cells in the presence of unlabeled SHBG. After 5 hr, approximately 11% of the tracer was associated with the cells, a process that was blocked by RAP (Figure 1C, filled columns). Megalin-mediated uptake of testosterone was critically dependent on the amount of SHBG present in the culture medium as shown by varying the molar ratio of carrier and testosterone (Figure 1C, open columns). At a ratio of carrier to steroid of 5:1 when 95% of the steroid is bound to SHBG (Figure S2), cell association of [^{3}H]testosterone was absolutely dependent on megalin activity as evidenced by RAP inhibitability. At a molar ratio of 1:10 when 98% of [^{3}H]testosterone is free (Figure S2), cell association was independent of megalin and insensitive to RAP. At a molar ratio of 1:1 when 20% of testosterone is complexed (Figure S2), uptake was partially dependent on megalin activity (Figure 1C). In the human circulation (adult males), the ratio of SHBG to testosterone is approximately 3:1 (Turner et al., 1984). SHBG bound testosterone was unable to enter cells when megalin was blocked by RAP and instead accumulated in the medium of the cells (Figure 1D).

To confirm that SHBG and androgens are taken up in a complex (and not independently of each other) by cells, we incubated BN16 cells with preformed complexes of dihydrotestosterone (DHT)/SHBG labeled either at the steroid ([^{3}H]DHT; Figure 1E, filled bars) or the carrier moiety ([^{125}I]SHBG; Figure 1E, open bars). Similar amounts of both tracers were detected in the cell fraction in a RAP-sensitive manner. The same finding was seen for testosterone/SHBG complexes (Figure 1E, striped and hatched columns). Uptake of androgen/SHBG complexes was also blocked by anti-megalin antiserum and by purified SHBG, confirming that the SHBG/ABP receptor megalin is responsible for steroid uptake (Figure S3). Megalin was equally important for the cellular uptake of androgens and estrogens, as demonstrated by identical RAP-sensitive uptake mechanisms for [^{3}H]DHT/SHBG and [^{3}H]17β-estradiol ([^{3}H]E2)/SHBG complexes in BN16 cells (Figure 1F). Remarkably, keratinocytes that do not express megalin were unable to internalize significant amounts of complexed DHT or E2 (Figure 1F). The different abilities of BN16 cells and keratinocytes to accumulate bound steroids were not due to differences in nuclear-hormone-receptor profile because neither cell type expressed detectable levels of androgen or estrogen receptors (Figure S1B).

We confirmed the intracellular delivery of bound sex steroids through megalin-mediated endocytosis using confocal fluorescence microscopy. When FITC-labeled DHT was complexed with SHBG and added to BN16 cells, uptake and intracellular colocalization of FITC-DHT and SHBG was shown (Figure 2A). Megalin was indispensable for this process, as cellular uptake of complexed FITC-DHT was blocked completely by RAP (Figure 2A). Complexed steroids internalized via megalin were fully active in inducing an androgen-responsive luciferase reporter construct transfected into BN16 cells. In fact, 1 nM of SHBG bound DHT was at least as active in inducing the reporter as unphysiologically high equimolar amounts of free steroids added to the culture medium (Figure 2B). SHBG alone had no effect (Figure 2B). RAP inhibited gene induction by SHBG-complexed but not by free DHT (Figure 2C), indicating the need for the megalin pathways for signaling of steroid/carrier complexes.

Taken together, our data in BN16 cells demonstrated that, under physiological conditions where 98%–99.5% of sex steroids are present in protein bound form (Dunn et al., 1981), endocytosis is quantitatively more relevant for tissue delivery of biologically active steroid hormones than free diffusion. To test this hypothesis in vivo, we evaluated the consequences of megalin deficiency

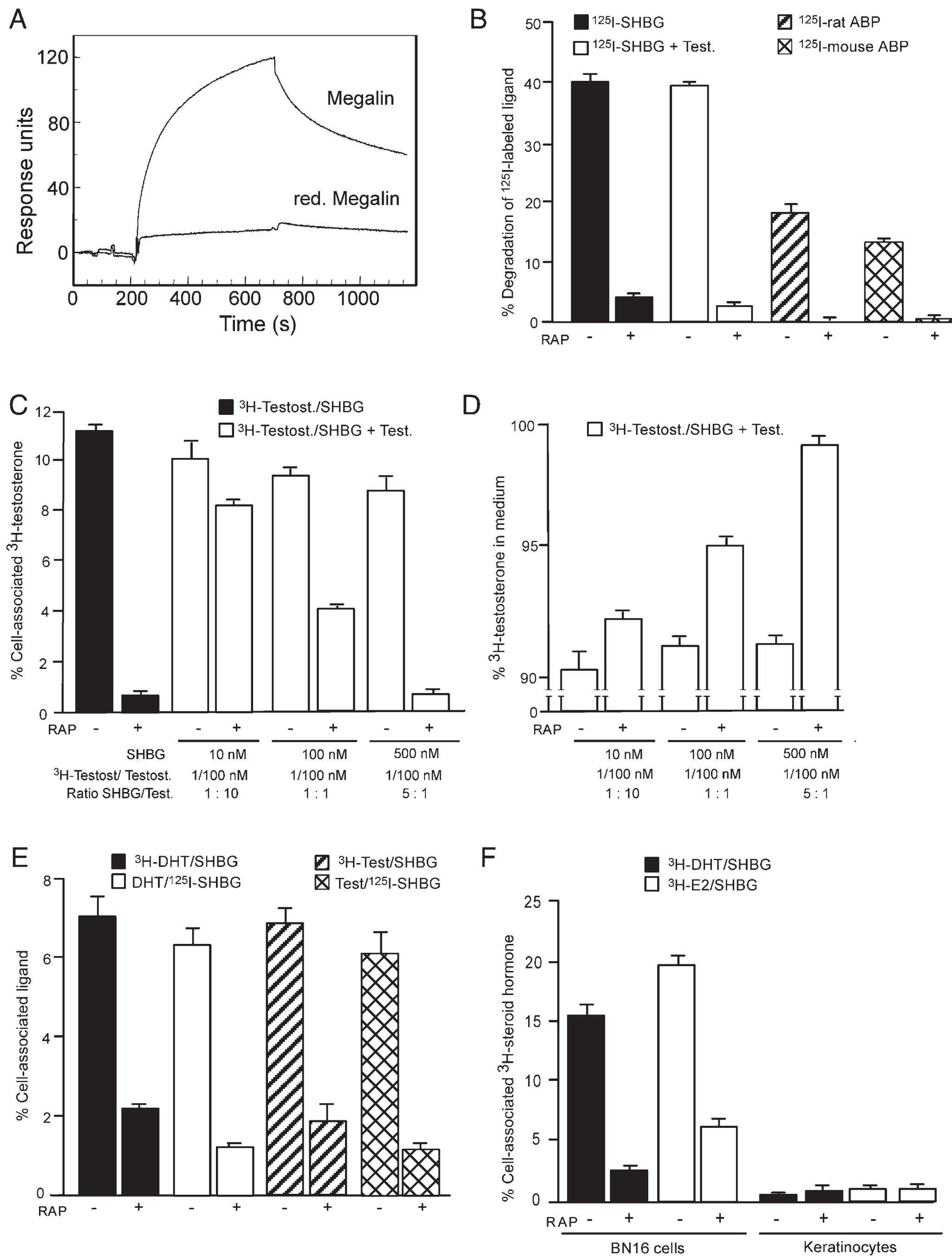

Figure 1. Cellular Metabolism of SHBG and SHBG/Steroid Complexes

All data represent mean values ± standard error of the mean (SEM).

(A) Surface plasmon resonance analysis of 500 nM recombinant SHBG binding to native but not reduced megalin immobilized on the sensor-chip surface.

(B) Triplicate monolayers of BN16 cells were incubated with 1 nM recombinant ^{125}I-labeled SHBG (with or without 1 nM testosterone), recombinant ^{125}I-labeled rat ABP, or recombinant ^{125}I-labeled mouse ABP in the presence (+) or absence (–) of the receptor-associated protein (RAP). The amount of iodinated carrier degraded after 5 hr was determined and indicated as % of total tracer added.

(C and D) BN16 cells were incubated with 1 nM [^{3}H]testosterone in the presence of 500 nM recombinant SHBG (filled columns) or with 1 nM [^{3}H]testosterone in the presence of 100 nM unlabeled testosterone and the indicated concentrations of SHBG (open columns). After 5 hr, the amount of [^{3}H]testosterone associated with the cells (C) or residing in the medium (D) in the presence or absence of RAP was determined.

(E) BN16 cells were incubated with 1 nM of the indicated preformed SHBG/androgen complexes for 5 hr in the presence or absence of RAP, and the amount of cell-associated tracer was determined thereafter.

(F) BN16 cells and human keratinocytes were incubated with 1 nM [^{3}H]DHT (closed columns) or 1 nM [^{3}H]17β-estradiol (E2) (open columns) in the presence of 10 nM recombinant SHBG for 5 hr, and the amount of tracer associated with the cells was determined in the presence or absence of RAP.

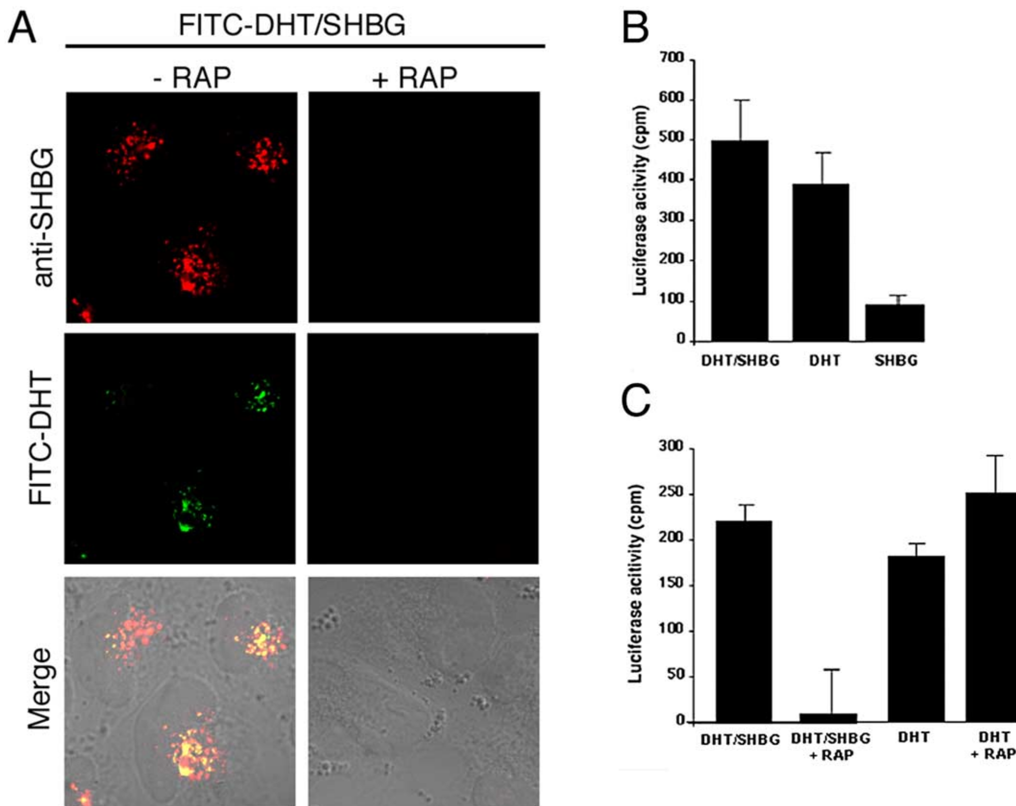

Figure 2. Uptake and Signaling by SHBG/DHT Complexes in BN16 Cells

(A) Cells were incubated with 800 nM preformed complexes of FITC-DHT/SHBG in the presence or absence of RAP, and the subcellular localization of FITC-DHT and SHBG (anti-SHBG antibody, followed by secondary Alexa 660-labeled IgG) was detected by confocal immunofluorescence microscopy.

(B) Induction of an androgen-responsive luciferase gene was evaluated in BN16 cells treated with 1 nM free (DHT) or 1 nM complexed DHT (DHT/SHBG) or with SHBG-conditioned medium only (SHBG).

(C) Luciferase gene induction by 1 nM DHT/SHBG or 1 nM free DHT in the absence or presence of 5 μM RAP. Data in (B) and (C) represent mean values ± SEM.

on the action of sex steroids using the *megalin* knockout mouse model (Willnow et al., 1996). Most megalin-deficient (*megalin*$^{-/-}$) mice die perinatally from a defect in forebrain development. However, approximately 5% of *megalin*$^{-/-}$ animals survive the perinatal period, enabling analysis of megalin deficiency in the adult organism (Nykjaer et al., 1999). In the following studies, *megalin* knockout mice on a hybrid (129/SvEmcTer × C57BL/6N) and on an inbred (CD1) genetic background were used. Both strains gave identical results. Thus, *megalin*$^{-/-}$ females were characterized by a closure of the vagina opening, a feature not seen in control littermates (Figure 3A). As a consequence of the closed vagina cavity, uterine fluid accumulated in the uterus of the knockouts, grossly inflating the organ (Figure 3B). In wild-types, megalin is expressed on the luminal surface of the uterine epithelium (Figure 3C; Figure S4), with onset of expression at puberty. Interestingly, megalin expression in the endometrium is tightly regulated during estrus cycle, with highest levels of expression in metestrus II and diestrus (Figure 3C). Loss of receptor expression in knockouts did not alter the overall tissue architecture that was composed of a mesenchymal stroma covered by a layer of epithelial cells. However, the knockout tissue appeared thinner compared to that of controls, likely due to the accrual of mucoid secretion that massively stretched the uterus (Figure 3D).

Female rodents are born with a closed vagina cavity that opens up 4 to 5 weeks after birth due to estrogen-induced apoptotic processes (Rodriguez et al., 1997). Application of anti-estrogens blocks this process and delays vagina opening, a defect that is used as a bioindicator of anti-estrogen action (Ashby et al., 2002; Chadwick et al., 1988). Thus, the phenotype in the megalin-deficient mice may be indicative of impaired estrogen signaling during the postnatal development of the female genitalia. Consistent with this hypothesis, the histological appearance of vaginal tissues in *megalin*$^{-/-}$ adult animals was similar to that of wild-type juvenile mice before puberty (Figures 3E–3K). Adult wild-type mice presented with a clearly defined vagina opening (Figure 3E) surrounded by a thick columnar epithelium (Figure 3F). In contrast, in adult megalin-deficient mice, the vagina opening was closed by a septum composed of an epithelial cell layer followed by connective tissue and skin (Figures 3G and 3H). Similar features were

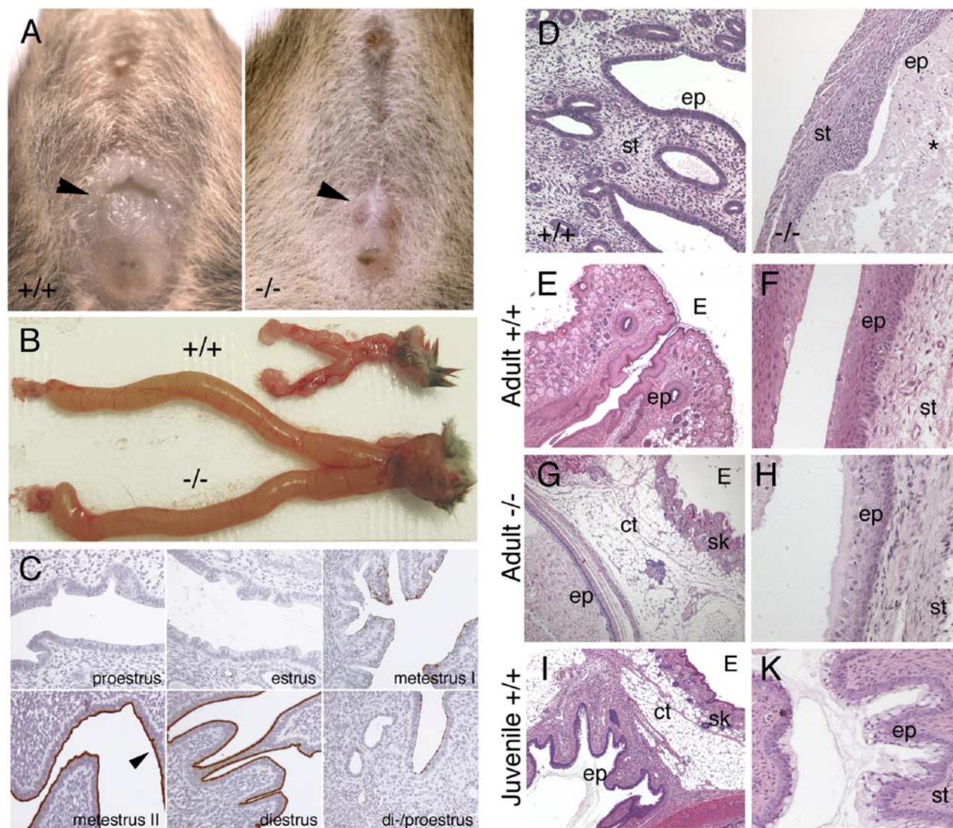

Figure 3. Female Reproductive Tracts in Wild-Type and Megalin-Deficient Mice

(A) Inspection of the external genitalia of female mice, with closure of the vagina cavity (arrows) in megalin-deficient (–/–) but not wild-type animals (+/+).

(B) Uteri of wild-type and *megalin*$^{-/-}$ mice.

(C) Expression of megalin (arrow) on the luminal surface of the uterine epithelium of wild-type mice during estrus cycle.

(D) Hematoxylin and eosin (H&E) stained sections of wild-type and megalin-deficient uteri in diestrus, indicating epithelial (ep) and stromal tissues (st), as well as mucoid accrual in the uterine lumen of the knockouts (asterisk).

(E–K) H&E-stained sections from vaginal tissues of 12-week-old wild-type (E and F) and megalin-deficient (G and H) animals in diestrus or from 2-week-old wild-type mice (I and K). (E), (G), and (I) depict cross-sections of the vagina opening, where E indicates the exterior of the genitalia. (F), (H), and (K) are higher magnifications of vaginal tissue sections. ct, connective tissue; ep, vaginal epithelium; sk, skin; st, stroma. (C, E, G, I, ×40; D, F, H, K, ×100.)

seen in wild-type juvenile mice prior to sexual maturation (Figures 3I and 3K). E2 levels in *megalin*$^{-/-}$ females were similar to the levels in wild-type littermates (Table 1), indicating that estrogen insensitivity rather than lack of estrogens was the underlying cause of this phenotype.

As well as suffering from estrogen insensitivity, megalin-deficient mice also lack proper androgen signaling

Table 1. Steroid-Hormone Concentrations in Adult Plasma or E14.5 Embryonic Tissue Extracts of Wild-Type and *megalin* Knockout Mice, ± Standard Error of the Mean

	+/+	n	–/–	n
Adult Plasma				
Testosterone (pg/ml)	6154.0 ± 2355	12	6440.0 ± 2442	6
DHT (pg/ml)	808.0 ± 245	12	978.0 ± 265	6
E2 (pg/ml)	41.5 ± 2.3	10	44.9 ± 10.2	6
E14.5 Embryo				
Testosterone (ng/g)	454.0 ± 46	19	800.0 ± 156*	10
DHT (ng/g)	388.0 ± 26	28	496.0 ± 55**	14
E2 (ng/g)	125.0 ± 43	11	102.0 ± 30	6

Testosterone and DHT levels were determined in males; E2 levels were determined in females. n, number of animals. *$p < 0.05$, Mann-Whitney U test; **$p < 0.05$, Student's t test.

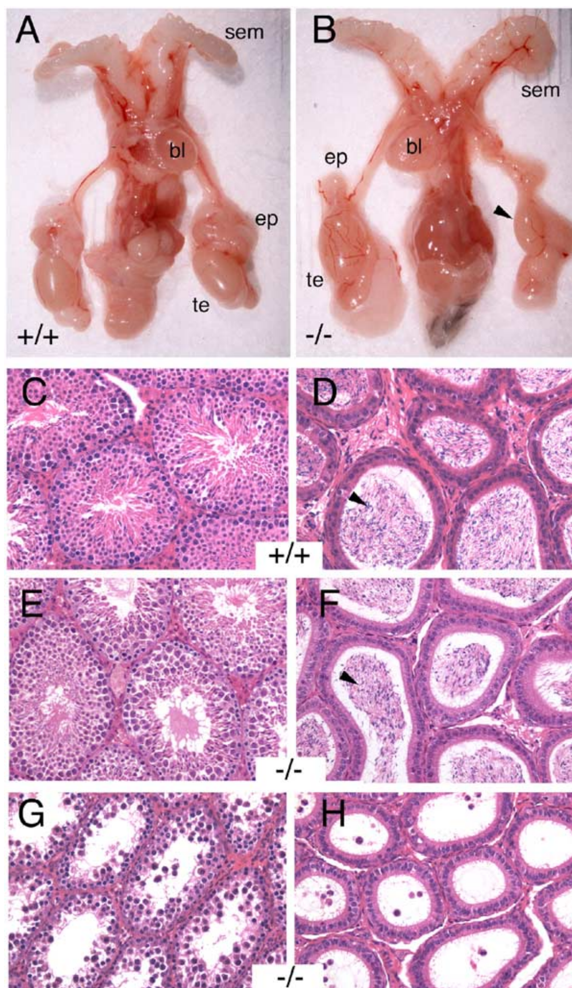

Figure 4. Male Urogenital Tracts of Adult Wild-Type and Megalin-Deficient Mice

(A and B) Inspections of the urogenital tracts of wild-type (A) and megalin-deficient adult mice (B). Note the reduced size of the cryptorchid testis in the *megalin*$^{-/-}$ animal (arrow).

(C–H) H&E-stained sections from wild-type testis (C) and corpus epididymis (D) compared to sections from descended (E) and nondescended (G) testis or descended (F) and nondescended (H) corpus epididymis of *megalin*$^{-/-}$ mice. Arrowheads indicate sperm in the lumen of wild-type (D) and descended *megalin*$^{-/-}$ epididymi. bl, bladder; ep, epididymis; sem, seminal vesicles; te, testis. (C–H, ×200.)

during development of the male reproductive organs. In all male receptor-deficient animals analyzed (n = 21), the left testis had not descended into the scrotum but was located in the body cavity (cryptorchidism). As a consequence of the unilateral testicular maldescent, the cryptorchid (left) testis was poorly developed and severely retarded in size (Figure 4B). Other aspects of the urogenitalia such as the contralateral testis, the epididymis, or the seminal vesicles exhibited normal appearance (Figure 4B). In histological sections, the cryptorchid testis was characterized by degenerated germinal epithelia of the seminiferous tubules and by the absence of germ cells (Figure 4G). The corresponding epididymis was devoid of sperm (Figure 4H). In contrast, the descended testis of knockout mice exhibited well-differentiated germinal epithelia (Figure 4E) and produced spermatozoa that filled the lumen of the epididymal ducts (Figure 4F), identical to the situation in wild-type controls (Figures 4C and 4D). Thus, megalin activity is not required for normal development and structural integrity of the male reproductive organs but is essential to control descent of the testes. Histological abnormalities in the cryptorchid testis are likely secondary to its exposure to a higher temperature in the abdomen.

Descent of the testes proceeds in two distinct steps. In early mouse embryos, the testes migrate from a position near the lower pole of the kidney to a region close to the bladder (transabdominal phase), while, after birth, the testes move further into the scrotum (inguinoscrotal phase) (Heyns and Hutson, 1995). Defects in either phase of testicular descent cause cryptorchidism (Toppari and Kaleva, 1999). To identify what phase of the testicular descent was affected in megalin-deficient mice, we analyzed the urogenital tract in E18.5 (data not shown) and newborn animals (Figure 5). In wild-type neonates, the testes were located near the bladder neck, consistent with completion of the transabdominal descent (Figure 5A). In contrast, in the *megalin*$^{-/-}$ newborns, both testes were positioned halfway between the kidney and the bladder (Figure 5A). Remarkably, an extensive ligament-like structure was apparent that fixed the gonads to the dorsal abdominal wall (arrow, Figure 5A). This structure is the cranial suspensory ligament (CSL), one of two tissues that control the position of the gonads in both sexes. The second tissue is the gubernaculum, a structure that attaches to the caudal end of the gonads. Both ligaments are present in male and female embryos around midgestation. In females, the CSL persists and holds the ovaries in a position close to the kidney. In males, the primordium of the CSL regresses during development, and the gubernaculum "tracks" the testes toward the lower abdomen (Heyns and Hutson, 1995). Histological examination of the urogenital tract in wild-type newborn males highlights this concept (Figure 5B). The testes and epididymi are located close to the bladder. The gubernaculum (composed of gubernacular cord and gubernacular bulb) is fully developed but the CSL is absent. In megalin-deficient newborns, both gubernaculum and CSL are still present at the lower and upper pole of the gonads, respectively (Figure 5C), with the CSL tethering the testes and epididymi to the abdominal body wall dorsal to the kidney (Figure 5D).

The descent of the testes is tightly regulated by androgens (Husmann and McPhaul, 1991; Spencer et al., 1991). Among other effects, androgens induce regression of the primordium of the CSL in male embryos starting around E14.5, enabling free movement of the gonads toward the lower abdomen (Emmen et al., 1998; Lee and Hutson, 1999). In male mice with an androgen receptor (AR) gene defect (Hutson, 1986; Zimmermann et al., 1999) or in rodent embryos treated in utero with the AR antagonist flutamide (van der Schoot and Elger, 1992), lack of fetal androgen signaling results in abnormal persistence of the CSL and in testicular maldescent. To confirm that *megalin*$^{-/-}$ male embryos also suffer from impaired induction of CSL regression, we

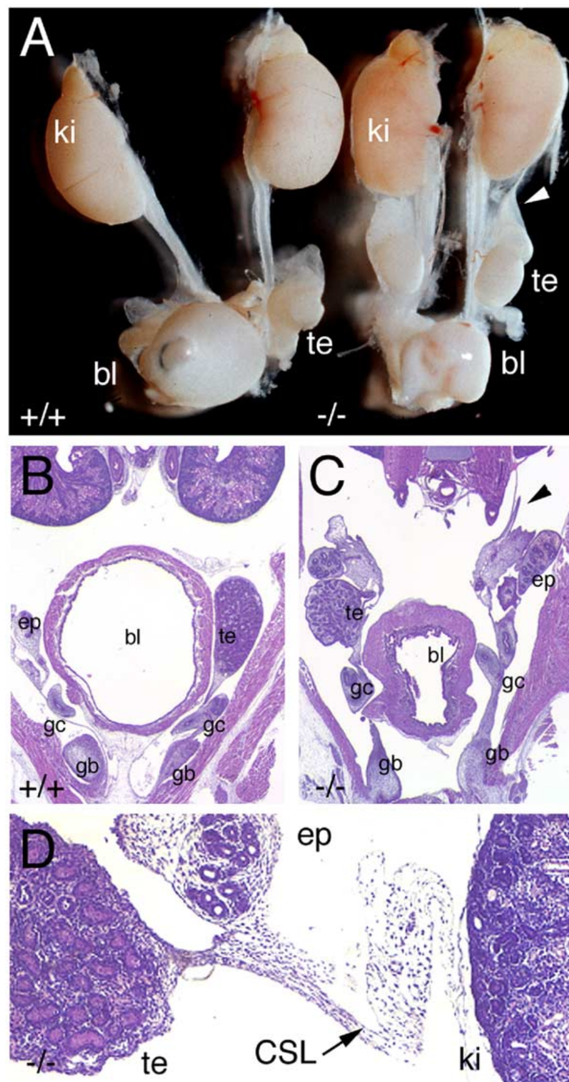

Figure 5. Urogenital Tracts of Male Neonates

(A) Urogenital tracts from wild-type and megalin-deficient newborn mice. The arrowhead highlights the cranial suspensory ligament (CSL) attached to the gonads in *megalin*$^{-/-}$ animals.

(B and C) Histological sections of the urogenital tracts of wild-type (B) and *megalin*$^{-/-}$ neonates (C) (magnification ×25). The arrow in (C) indicates the CSL fixing the gonads of *megalin*$^{-/-}$ animals to the dorsal abdominal body wall.

(D) Higher magnification (×100) of the CSL in *megalin*$^{-/-}$ neonates. bl, bladder; ep, epididymis; gb, gubernacular bulb; gc, gubernacular cord, ki, kidney; te, testis.

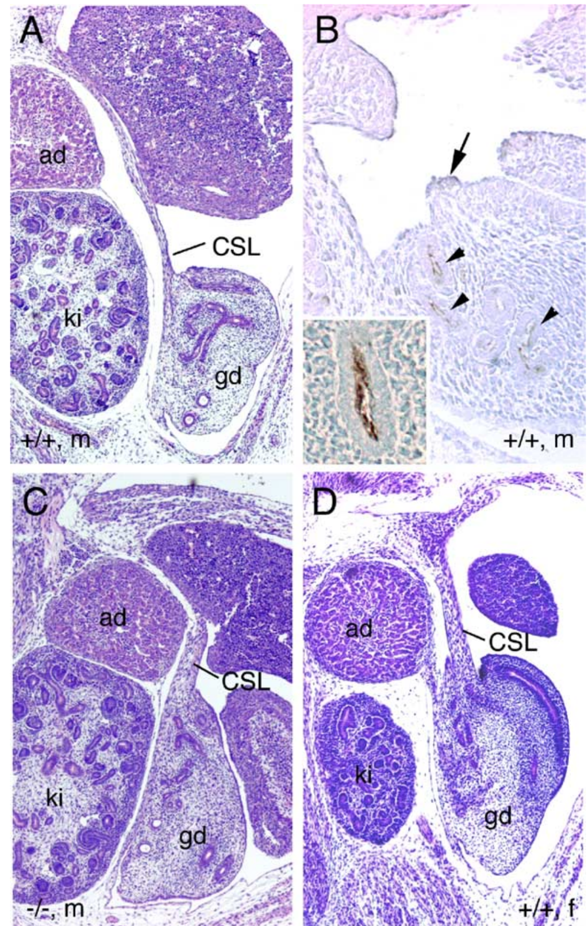

Figure 6. Histology and Megalin Expression Pattern of E14.5 Urogenital Tracts

(A and B) Histological sections from wild-type male embryos subjected to staining with H&E (A) or immunodetection of megalin (B). Arrowheads in (B) indicate expression of megalin in the mesonephric tubules of the gonads (highlighted in inset). The arrow marks the primordium of the CSL.

(C) H&E section of the urogenital tract of a male E14.5 *megalin*$^{-/-}$ embryo.

(D) Section of the urogenital tract of a wild-type female E14.5 embryo. ad, adrenal gland; gd, gonads; ki, kidney. (A, C, D, ×40; B, ×200).

analyzed the appearance of this ligament in wild-type and knockout embryos. In wild-type E14.5 male embryos, the CSL was still apparent as a thin tissue strand that connected the gonads with the craniolateral surface of the dorsal abdominal wall (Figure 6A). At this time in development, megalin was expressed in the mesonephric tubules of the gonads in close proximity to the primordium of the CSL (Figure 6B). In receptor-deficient E14.5 males, the CSL represented a thick cord-like structure (Figure 6C) that shared striking resemblance with the CSL of wild-type female embryos (Figure 6D). At earlier time points in development, no obvious difference in the histological appearance of wild-type and *megalin*$^{-/-}$ urogenital tissues was seen (data not shown). Failure of regression of the CSL in megalin-deficient male embryos was not due to a defect in androgen production. Rather, the amount of circulating testosterone and DHT was increased 2-fold compared to control littermates (Table 1). Also, no discernable difference was seen in androgen levels in adult *megalin*$^{-/-}$ males compared to controls (Table 1).

Presently, genes regulated by steroid hormones during induced regression of the CSL are unknown. To identify such genes and to evaluate their expression levels in megalin-deficient mice, we performed global gene-expression profiling on gonads from E14.5 *megalin*$^{+/+}$ and *megalin*$^{-/-}$ embryos. A total of six genes were

marked as significantly changed according to the stringent preset quality criteria (expression levels > 200, fold change > 2) (Table S1, upper panel). Five out of the six genes indicated defects in testis development (*Tex12*, *Morc*, *Stk25*) as well as impairment in steroid-hormone signaling (*Ramp2*, *Mpo*) in the knockouts. Remarkably, *Ramp2*, a gene induced by sex steroids, was reduced in the knockouts, whereas *Mpo*, a sex-steroid-repressed gene, was induced. We also evaluated the expression profiles with respect to 20 candidate genes known to be upregulated in the adult mouse epididymis upon androgen removal (Chauvin and Griswold, 2004) (Table S1, lower panel). Intriguingly, megalin-deficient embryos exhibited significantly increased expression of *Igfbp5*, a gene that is induced upon androgen removal in mice (Chauvin and Griswold, 2004) and that is upregulated in patients with androgen insensitivity syndrome (Diesing et al., 2003).

Finally, we confirmed steroid insensitivity of megalin-deficient embryonic tissues using exogenous androgen application. When pregnant rats are injected with high doses of androgens, excessive androgen signaling causes aberrant regression of the CSL in the female embryos (Emmen et al., 1998), a phenomenon that we confirmed in the mouse model (Figure 7). Normally, the ovaries of wild-type and megalin-deficient female E18.5 embryos are positioned laterally to the lower kidney pole (Figures 7A and 7B), with the CSL fixing the gonads to the dorsal body wall (Figures 7E and 7F). When treated with exogenous DHT in utero, wild-type females induced regression of the CSL (Figure 7G), displacing the ovaries from the lower kidney pole (Figure 7C). In contrast, megalin-deficient females responded poorly to DHT injection, with only partially displaced ovaries (Figure 7D) and clearly visible CSL (Figure 7H). In untreated wild-type male embryos, the gonads are located near the bladder in wild-types (Figures 7I and 7N) but tethered through aberrant CSL to the body wall dorsal to the kidney in knockouts (Figures 7K and 7O). Application of exogenous DHT did not affect positioning of the gonads in wild-type males because endogenous androgen signaling is normal in these animals (Figures 7L and 7P). Similar to the females, megalin-deficient male embryos did not respond properly to exogenous androgen application and still exhibited abnormal persistence of the CSL (Figure 7Q) and maldescent of the gonads (Figure 7M). Similar findings were obtained by application of DHT propionate or testosterone (data not shown).

In conclusion, our findings in mice lacking megalin uncovered insensitivity to androgen and estrogen signaling as well as distinct defects in fetal and postnatal maturation of the reproductive organs. These phenotypes are highly reminiscent of defects induced by anti-androgens and anti-estrogens in rodents and strongly implicate this receptor pathway for SHBG/ABP in sex-steroid action in vivo.

Discussion

Until now, the identity and the role of proposed SHBG/ABP receptors in steroid-hormone metabolism has remained obscure. We show that megalin is an endocytic receptor for SHBG/ABP in steroid target tissues that mediates uptake of carrier bound androgens and estrogens. When exposed to sex steroids in the presence of physiological concentrations of carrier proteins, cells are critically dependent on megalin activity to internalize significant amounts of the hormones. Little uptake is seen in the presence of megalin antagonists (Figure 1C) or in keratinocytes that lack receptor expression (Figure 1F). Bound steroids are delivered to intracellular compartments (Figure 2A) and act as inducers of steroid target genes, the hallmark of cellular steroid action (Figures 2B and 2C). Receptor interaction is solely dependent on the recognition of the carrier moiety by megalin and is independent of the presence of the steroid (Figure 1B). Although the exact route for internalization and intracellular delivery of sex steroids is still unclear, it likely involves vesicular pathways similar to those involved in endocytosis and intracellular trafficking of vitamin D metabolites (Wu et al., 2000; Nykjaer et al., 2001).

Among other work, experimental support for the free hormone hypothesis was obtained by studies where uptake of sex steroids was evaluated in cultured cells. In these experiments, steroids entered cells when added in free form to the medium but were blocked from cellular entry in the presence of SHBG (Damassa et al., 1991; Giorgi and Stein, 1981). These findings led to the concept that only free steroids can access cells. Thus, many studies on the cellular metabolism of steroid hormones were performed in the absence of carrier proteins or using hormone derivatives that do not interact with carriers. While these experimental conditions are useful to investigate intracellular aspects of steroid-hormone activity, they have drawn attention away from the physiological context where, depending on the steroid, as much as 95%–99.9% of the metabolites are complexed to proteins (Dunn et al., 1981). Under these conditions, free diffusion may be of quantitatively lesser importance for cellular delivery of steroids, in particular in tissues that are in demand of large amounts of steroid hormones. Contrary to the aforementioned studies, we demonstrate that cells are able to internalize complexed sex steroids and to induce steroid target genes, provided that they express the SHBG/ABP receptor megalin. Although megalin is abundantly expressed in several absorptive epithelia in vivo, few established cell lines express the receptor. Thus, this SHBG/ABP uptake pathway may have been missed in previous studies using cells lacking the receptor.

Evidence for a physiological role of megalin in sex-steroid action stems from the defects observed in the receptor-deficient mouse model. Apart from bone disease as a consequence of hypovitaminosis D (Nykjaer et al., 1999), adult *megalin*$^{-/-}$ animals suffer from anomalies in genital maturation consistent with insensitivity to androgens and estrogens. Despite normal (E2) or even increased levels (testosterone, DHT) of circulating hormones, these mice exhibit defects seen in rodents treated with anti-androgens and anti-estrogens. Thus, female receptor null mice fail to induce opening of the vagina cavity, a benchmark of natural puberty. This process can be induced in the immature rodent by injection of E2 and blocked by application of anti-estrogens

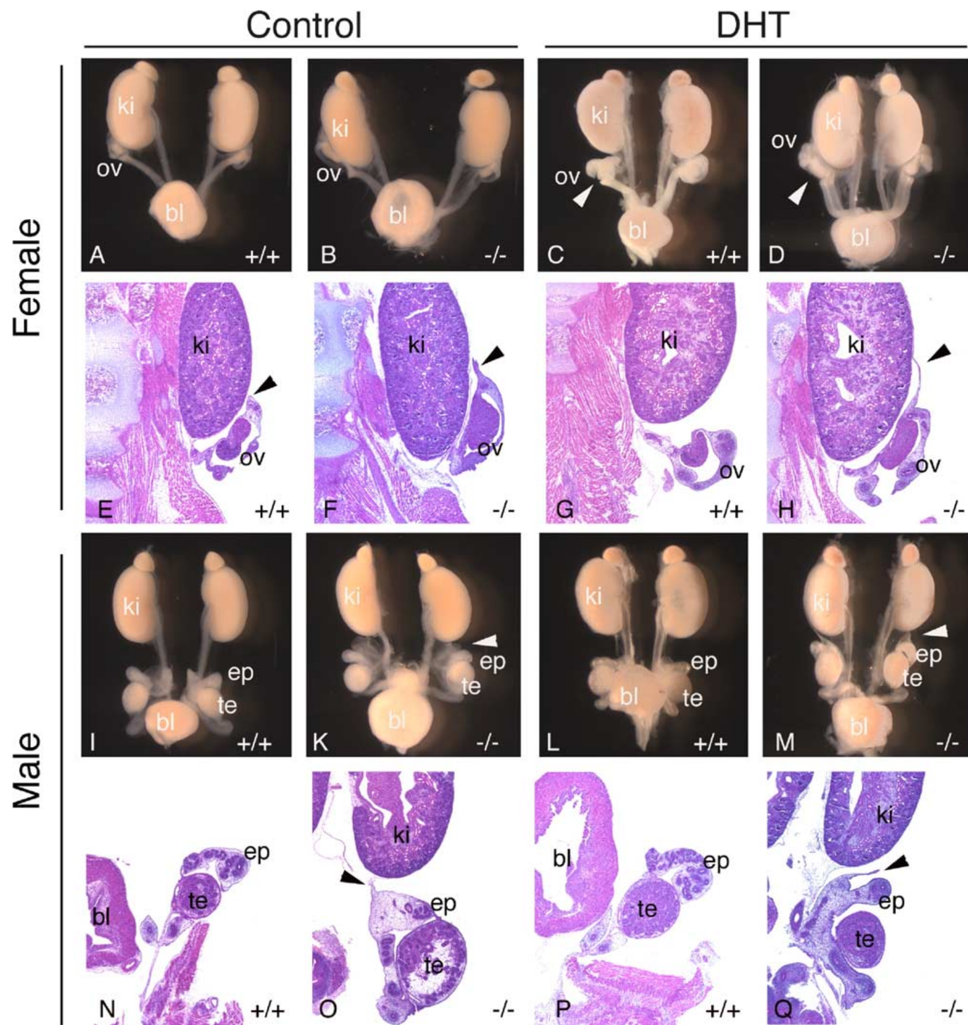

Figure 7. Urogenital Tracts of E18.5 Embryos Treated with DHT In Utero

Mouse embryos were treated in utero with buffer (control) or DHT solution from E10.5 to E18.5 (see Experimental Procedures for details), and the urogenital tracts of female (A–H) and male (I–Q) animals were studied by external inspection and histology at E18.5. A total of 8 to 20 animals were analyzed per group. In wild-type and megalin-deficient females, the ovaries (ov) are positioned laterally to the lower kidney pole (A and B), with the CSL fixing the gonads to the abdominal body wall (arrows in [E] and [F]). When treated with DHT, the CSL in wild-type females regresses (G), displacing the ovaries from the lower kidney pole (arrow in [C]). Megalin-deficient females exhibit marginally displaced ovaries (arrows in [D] and [H]). In the male sex, the gonads are located near the bladder in wild-types (I and N) but are tethered through the CSL to the abdominal body wall close to the kidneys in *megalin* knockouts (arrows in [K] and [O]). Similar findings can be seen in wild-type (L and P) and *megalin*$^{-/-}$ embryos (M and Q) treated with exogenous DHT. bl, bladder; ep, epididymis; ki, kidney; ov, ovaries; te, testis.

such as ICI 182,780 (Ashby et al., 2002). Presently, the small number of megalin-deficient females available (n = 18) precluded rigorously testing whether these mice respond to exogenous E2 application. However, in three such animals tested, injection of E2 did not induce vagina opening.

Similar to females, *megalin*$^{-/-}$ males also exhibit defects indicative of sex-steroid insensitivity, namely unilateral testicular maldescent. This process can be induced in offspring of pregnant rats and mice treated with flutamide, an inhibitor of the AR, or with finasterite, a blocker of 5α reductase that converts testosterone to the more potent androgen DHT (Spencer et al., 1991). Many hypotheses about the mechanisms of hormonally controlled descent of the testes have been advanced, none of which have been proven definitively. This difficulty is in part due to the mechanistical differences that exist between humans, rodents, and other mammals in this process. Common to all species is the pivotal role of the CSL and the gubernaculum in determining positioning of the gonads in the body (Heyns and Hutson, 1995; Husmann and McPhaul, 1991; Spencer et al., 1991). Much of the focus has been placed on the development of the gubernaculum in males as it guides the migrating testes through the inguinal canal into the scrotum. Gene defects of several proteins, including HOXA-10 (Rijli et al., 1995) or insulin-like hormone 3 (Nef and Parada, 1999; Zimmermann et al., 1999), that impair development of the gubernaculum cause maldescendus testis. Recently, a role of hormonally regu-

lated development of CSL also received major attention. Involution of the CSL in males is androgen dependent, and, in mice lacking the AR (Hutson, 1986; Zimmermann et al., 1999) or in animals treated with anti-androgens (van der Schoot and Elger, 1992), the CSL persists. Both bi- and unilateral testicular maldescent may be observed, highlighting the concept of asymmetry in urogenital development (Lee and Hutson, 1999; van der Schoot and Elger, 1992). Unilateral testicular maldescent as a consequence of persistence of the CSL is a feature also shared by the *megalin* knockout mouse. Regression of the CSL cannot be induced by exogenous application of high levels of free androgens (Figure 7), strongly supporting the concept that endocytic uptake of complexed steroids rather than free diffusion of unbound steroids is required to specifically deliver them to their target tissues.

Assuming a pivotal role of megalin in sex-steroid signaling, it is intriguing to note that megalin null mice do not share all of the anomalies seen in mice deficient for the AR (e.g., testicular feminization) (Couse and Korach, 1998; Hutson, 1986) or estrogen receptors (ER) (e.g., uterine hypoplasia) (Couse et al., 1999; Lubahn et al., 1993). This observation suggests that megalin deficiency impairs spatially and temporarily restricted activities of androgens and estrogens in line with the distinct expression pattern of the receptor during genital development. In particular, activities that require apoptotic processes induced by paracrine action of steroid hormones may involve this receptor pathway. Thus, the exact molecular mechanism causing E2-induced opening of the vagina cavity is unclear at present, but it involves paracrine processes through tissues other than the vaginal epithelium itself. This hypothesis is based on the finding that blocking apoptosis in mice by overexpression of Bcl2 in epithelial and subepithelial cells of the vaginal mucosa results in vaginal obstruction, whereas Bcl2 expression in epithelial cells alone is insufficient (Rodriguez et al., 1997). Similarly, local paracrine processes are also responsible for regulation of testicular descent. Although androgens play a causal role in this process, the hormones likely do not act on the CSL directly, as this tissue does not express detectable levels of AR (Emmen et al., 1998; Hutson et al., 1994). Instead, androgens are believed to act on neighboring cell types (e.g., primordium of the CSL) that release paracrine factors such as epidermal growth factor, which has been shown to ameliorate the effects of flutamide application on descent of the testis (Cain et al., 1994). The concept of paracrine action of steroids during testicular descent and vagina opening may explain why expression of megalin is seen not in the cell types that undergo apoptosis (CSL, vaginal epithelium) but in adjacent tissues such as mesonephric tubules and uterine epithelium (Figures 3C and 6B).

As well as acting in a locally restricted manner, steroid-induced opening of the vagina cavity and testicular descent are developmental processes that are induced during a very narrow time window in development, warranting the need for efficient steroid-uptake processes. This is best exemplified by the application of DHT analogs in pregnant rats to induce aberrant regression of the CSL in female embryos. In this model, involution of the CSL is seen when steroids are applied before E17 but not when they are applied at later embryonic stages (Emmen et al., 1998). Because AR expression in rats starts around E16.5, this leaves a narrow time window for androgen action on the CSL. Similarly, application of flutamide showed maximal effect on testicular descent only when applied between E16 and E17 (Husmann and McPhaul, 1991), a time when the AR is expressed in the mesenchymal and epithelial cells of the mesonephric tubules (where megalin is also seen).

In conclusion, our studies have uncovered endocytic pathways for cell-type-specific uptake of protein bound androgens and estrogens that are required for steroid-dependent maturation of the reproductive organs. Our findings provide a novel paradigm for a biological role of carrier bound steroids in hormone action that may change current concepts in steroid-hormone biology.

Experimental Procedures

Materials

His_6-tagged human SHBG, His_6-tagged murine and rat ABP, and GST-tagged rat RAP were purified from HEK293 EBNA cells (Hilpert et al., 2001) or bacteria (Nykjaer et al., 1999). Tritiated testosterone (TRK921), dihydrotestosterone (DHT) (TRK443), and 17β-estradiol (TRK322) were purchased from Amersham Pharmacia. FITC-labeled 1α-aminohexyl-17β-hydroxy-5α-androstan-3-one (FITC-DHT) was synthesized as published (Metzger et al., 2003).

Animals

The generation of mice with *megalin* gene inactivation has been reported before (Willnow et al., 1996). The line was kept by breeding of animals heterozygous for the receptor-gene defect (*megalin*$^{+/-}$). Studies were performed in megalin-deficient mice and their control littermates. The estrus cycle of the animals was predicted based on the composition of vaginal smears (Vianney, 1965) or the histological appearance of the mucous membranes formed by the vaginal epithelium (Krinke, 2004; Yuan and Carlson, 1987). For application of exogenous androgens, pregnant mice were injected subcutaneously with a daily dose of 2 mg of DHT (Sigma, Taufkirchen, Germany), DHT propionate (Steraloids, Newport, Rhode Island), or testosterone (Sigma) in 20 μl EtOH/180 μl sunflower oil from day 10.5 to 18.5 postcoitum of pregnancy (or with EtOH/oil only as control), and the embryos were removed for analysis at E18.5.

Immunohistological Analysis

Urogenital tracts from embryos, neonates, or adult mice were dissected, fixed overnight in 4% paraformaldehyde in PBS at 4°C, and embedded in paraffin. Routine paraffin sections were cut at 5 μm thickness and stained with hematoxylin and eosin. For megalin detection, unstained sections were incubated with sheep anti-megalin IgG (1:50,000) followed by peroxidase-conjugated rabbit anti-sheep antibody (1:100; Dako, Hamburg, Germany) and detection with diaminobenzidine.

Endocrine Analysis

Testosterone, DHT, and 17β-estradiol levels in adult plasma samples or E14.5 whole-embryo homogenates were determined using specific RIA (ICN Pharmaceuticals, Costa Mesa, California) or ELISA (for DHT; IBL, Hamburg, Germany) according to the manufacturers' recommendations following tissue extraction of the steroids by methanol and solid-phase extraction procedures (Nykjaer et al., 1999).

Gene-Expression Profiling

Gene-expression profiling was performed on pools of total RNA extracted from the gonads of five wild-type and five megalin-deficient E14.5 male embryos using the Mouse Genome 430 2.0 probe array (Affymetrix) according to the manufacturer's protocols. For

array-to-array comparison, the readings were scaled to a global intensity of 200 (GCos v1.2). The readings from the quantitative scanning were analyzed by the Affymetrix Gene Expression Analysis Software (DMT v3.0).

Cell Binding and Uptake Studies

Surface plasmon resonance analysis of recombinant human SHBG binding to megalin was performed according to standard protocols (Nykjaer et al., 1999). Cell uptake and lysosomal degradation of ^{125}I-labeled carrier proteins was determined as described (Nykjaer et al., 2001). For preformation of complexes, purified carrier and sex steroids were mixed in DMEM and incubated for 1 hr at room temperature prior to isolation of complexes over PD-10 columns (Amersham Pharmacia). Alternatively, complex formation was achieved by adding recombinant SHBG and steroids at a molar ratio of 10:1 to the cell medium. Cellular uptake of labeled steroids or SHBG was determined as cell-associated radioactivity present after washing of the cells with PBS and was expressed as percentage of the total amount of radioactivity added. For detection of DHT uptake, 1 μM of SHBG and 5 μM of FITC-DHT were incubated overnight at 4°C in DMEM containing 0.1% ovalbumin (binding buffer) and subsequently dialyzed against DMEM for 2 hr. BN16 cells were preincubated for 2 hr in DMEM prior to addition of 800 nM FITC-DHT/SHBG complexes in the presence or absence of 4 μM RAP in binding buffer. After incubation for 90 min at 37°C, the cells were washed, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and incubated with rabbit anti-SHBG IgG (1:400) and anti-rabbit Alexa 660 IgG (Molecular Probes, Europe BV). Uptake of SHBG and FITC-DHT was evaluated by confocal fluorescence microscopy.

Cell Signaling Studies

A reporter plasmid was constructed containing expression cassettes for the human AR driven by the CMV promoter and the luciferase gene controlled by the androgen-responsive MMTV promoter (provided by A. Brinkmann, Erasmus University). Because BN16 cells are notoriously difficult to transfect, biolistic procedures were used to transiently introduce the reporter construct (Bio-Rad Biolistic PDS-1000/He device; Bio-Rad, Sundbyberg, Sweden). Transfection efficiencies of approximately 1% were achieved. Eighteen hours posttransfection, the cells were incubated in DMEM, 0.1% ovalbumin in the presence of 1 nM DHT, conditioned media from HEK293 cells producing SHBG, SHBG media plus 1 nM DHT, or SHBG media plus 1 nM DHT plus 5 μM RAP. After 16 hr incubation, cells were washed, and cell lysates were generated using Reporter Lysis Buffer (Luciferase Assay System; Promega, Madison, Wisconsin). Twenty microliters of lysate was added to 100 μl of Luciferase Assay Reagent and quantified by luminometry. Determinations were performed in triplicate, and luciferase activity was expressed as total activity subtracted by the value for control incubation without DHT (approximately 150 cpm).

Supplemental Data

Supplemental Data include four figures and one table and can be found with this article online at http://www.cell.com/cgi/content/full/122/5/751/DC1/.

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Accession Numbers

Expression data described herein are available from ArrayExpress (www.ebi.ac.uk/arrayexpress) under the accession number E-MEXP-352.