Regulation and Ligand-binding Specificities of Two Sex-specific Bile Acid-binding Proteins of Rat Liver Cytosol*

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Rat liver cytosolic proteins were photoaffinity labeled with the synthetic steroid [3H]methyltrienolone in order to identify and characterize hepatic proteins that may participate in the intracellular binding and transport of steroid hormones and other steroids. A male-specific and a female-specific sterol-binding protein (SBP) that migrated to the 4 S region of a sucrose gradient and had similar molecular weights (male-specific 34 kDa protein (SBP34), female-specific 31 kDa protein (SBP31)) were thus identified. Experiments were undertaken to determine the biochemical basis for the sex-specific expression of these two proteins. In vivo hormonal manipulations established that the female-specific expression of SBP31 could, in part, be accounted for by the suppressive effects of androgen on SBP31 levels in male rats. In contrast, androgen stimulated expression of the male-specific SBP34, while estrogen and the estrogen-regulated continuous plasma growth hormone profile that is characteristic of adult female rats were suppressive toward this protein. Unlike several other androgen-dependent hepatic proteins, however, SBP34 did not require an intact pituitary for androgen-stimulated expression, nor was its expression stimulated by the intermittent pulses of plasma growth hormone that are characteristic of adult male rats. SBP34 and SBP31 were not induced but were suppressed to various extents by dexamethasone, phenobarbital, and clofibrate, drugs that are known to induce other hepatic proteins involved in steroid binding and metabolism. Competition experiments revealed that SBP31 has a relatively broad ligand specificity, with significant competition for [3H]methyltrienolone binding exhibited by bile acids (cholenoxycholonic acid and lithocholic acid) and a range of steroid hormones (progesterone, estradiol, testosterone, and 5a-dihydrotestosterone) when present in the low micromolar range. No binding was detected with this protein toward cholesterol, triaciniolone acetinone, 5a-androstane-3β,17β-diol, cholic acid, and deoxycholic acid. In contrast, SBP34 exhibited greater binding specificity, with competition for [3H]methyltrienolone binding observed only with primary bile acids (cholic acid and chenoxycholonic acid) and their metabolites (deoxycholic acid and lithocholic acid). On the basis of these binding specificities and the relatively high concentration of bile acids found in the liver, it is proposed that SBP31 and SBP34 function in the intracellular binding and/or transport of bile acids.

The liver is a major site for both the anabolic and catabolic metabolism of sterols including cholesterol, steroid hormones, and bile acids. For many of these sterols, extra- and intracellular transport is mediated by carrier proteins. For example, cholesterol is transported to the liver bound to plasma lipoproteins, its entry into the liver is mediated by specific cell surface receptor proteins, and its synthesis in liver is dependent upon precursor-specific carrier proteins (Scallen et al., 1974; Gill et al., 1985). Proteins have also been identified that function in the plasma transport, hepatic influx, and hepatic efflux of steroid hormones and bile acids (Petra et al., 1988; Ananthanarayanan et al., 1988; Ruetz et al., 1987). In contrast, intracellular transport processes for these compounds are poorly understood and the proteins that mediate intrahepatic transport, although inferred experimentally (Myant and Mitropoulos, 1977; Kitani and Kanai, 1981; Stolz et al., 1989; Answer et al., 1976), remain largely uncharacterized.

Although sterol-binding activity has been demonstrated in cytosolic preparations from liver and other tissues, in many cases it has been difficult to ascertain whether the observed binding activity represents cytosolic receptor proteins or proteins that elicit other hepatic proteins involved in steroid binding and metabolism. Competition experiments revealed that SBP31 has a relatively broad ligand specificity, with significant competition for [3H]methyltrienolone binding exhibited by bile acids (cholenoxycholonic acid and lithocholic acid) and a range of steroid hormones (progesterone, estradiol, testosterone, and 5a-dihydrotestosterone) when present in the low micromolar range. No binding was detected with this protein toward cholesterol, triaciniolone acetinone, 5a-androstane-3β,17β-diol, cholic acid, and deoxycholic acid. In contrast, SBP34 exhibited greater binding specificity, with competition for [3H]methyltrienolone binding observed only with primary bile acids (cholic acid and chenoxycholonic acid) and their metabolites (deoxycholic acid and lithocholic acid). On the basis of these binding specificities and the relatively high concentra-
ment with drugs and other xenobiotics (Waxman, 1986). Induction of these enzymes can facilitate drug activation and inactivation as well as the detoxification and excretion of toxic compounds. Steroid-binding proteins might also participate in the binding of lipophilic drugs and other foreign compounds and therefore may be responsible to drug exposure.

In the present study, we have utilized the synthetic androgen [3H]methyltrienolone in conjunction with photoaffinity labeling techniques to identify hepatic proteins that may function in intracellular steroid transport. A detailed characterization of the localization, regulation by hormones and drugs, and steroid-binding specificities of a male-specific 34-kDa protein and a female-specific 31-kDa protein is presented. Although these proteins were identified by photoaffinity labeling with an androgen, ligand competition experiments revealed that they exhibit greater binding affinity toward bile acids, suggesting that they may serve as intracellular bile carrier proteins.

MATERIALS AND METHODS

Animals—Fischer 344 rats, purchased from Taconic Labs, Germantown, NY, were used in all experiments except as indicated. Hypophysectomy, castration, and ovariec- tomy were performed by the supplier at 8 weeks of age and the rats were shipped at 9 weeks. Animals were held for 1–2 weeks after arrival before initiating any treatments. Hypophysectomy was judged complete by the absence of significant weight gain during this period and by the loss of hepatic NADPH cytochrome P-450 reductase activity (Waxman et al., 1989b).

Drug and hormone treatments were administered as follows (n = 3–5 individual animals/group). Pimecrolimus (Sigma) was supplied to rats in drinking water at 1 g/liter for 6 days. Dexamethasone (Sigma) was injected intraperitoneally, in corn oil, daily at 100 mg/kg for 4 days while clofibrate (Sigma) was injected intraperitoneally in corn oil, daily at 400 mg/kg for 3 days. Rats were killed 1 day after the last injection. The synthetic androgen methyltrienolone (Du Pont-New England Nuclear) was administered to rats for 7 days at a daily dose of 0.625 mg/kg, intraperitoneally, as described previously (LeBlanc and Waxman, 1988). In the case of the ovariec- tomized female Sprague-Dawley rats in Fig. 1, C and D, methyltrienolone was administered for 5, 8, or 15 days at a daily dose of 0.25 or 0.625 mg/kg. These in vivo treatments were kindly performed by Drs. Richard Janeczko and Milton Adesnik, New York University Medical Center. Human growth hormone (2.4 IU/mg, National Hormone and Pituitary Program) prepared as described previously (Waxman et al., 1988) was injected intraperitoneally for 1 week either by twice daily subcutaneous injections (7:00 a.m./7:00 p.m.) at 30 mg/150-g rat injection, or by continuous infusion using an Alzet osmotic minipump (Also Corp.) implanted subcutaneously and pumping at a rate of 0.63 µg of growth hormone/rat/h. This dose and protocol for continuous growth hormone treatment has been shown to fully feminize adult male rats with respect to several sex-dependent hepatic cytochrome P-450s (Waxman et al., 1990). Diethylstilbestrol (Sigma) was dissolved in ethanol, diluted 10-fold with propylene glycol, and the ethanol was then evaporated under a stream of nitrogen. The propylene glycol solution was infused into the rats for 1 week using osmotic mini-pumps implanted subcutaneously and pumping at a rate of 1.0 µg of diethylstilbestrol/rat/h.

Rats were killed by cervical dislocation following asphyxiation under CO2. Livers were quickly excised, minced in ice-cold 1.15% KCl, frozen in liquid nitrogen, then stored at −80 °C. Cytosols were prepared by homogenizing the frozen tissue in 5 volumes of cytosol buffer (10 mM Hepes, pH 7.4, 1.0 mM EDTA, 1.0 mM dithiothreitol, 25 mM sodium molybdate, 10% glycerol). Homogenates were then centrifuged at 100,000 × g for 60 min. Resulting supernatants (cytosols) were diluted with cytosol buffer to a protein concentration of 10 mg/ml, aliquoted, and stored at −80 °C. Protein concentrations were measured according to Bradford (1976) using commercially available reagent (Bio-Rad) and bovine serum albumin as standard.

Identification of Steroid-Binding Proteins—Cytosol (100 µg of protein) was incubated for 2 h with 120 nm [3H]methyltrienolone (87 Ci/mmol, Du Pont-New England Nuclear) in cytosol buffer containing no glycerol (PL buffer) in a total volume of 57 µl. Cytosolic methyltrienolone-binding proteins were then photoaffinity labeled using an apparatus similar to that described by Katzenellenbogen et al. (1974). Essentially, a 450-watt mercury vapor lamp was suspended in a glass chamber equipped with a cooling jacket of circulating water (4 °C). This chamber was inserted into a larger glass container filled with a solution of saturated copper sulfate, which served to filter emitted wavelengths <315 nm. The entire apparatus was immersed into the center of a bath of ethylene glycol/water (50:50) that was maintained at 4–6 °C by a jacket of circulating methanol (0 °C). Incubation tubes were suspended into this bath in positions surrounding the lamp and the samples irradiated for 10 min. Preliminary experiments revealed that photoaffinity-labeled protein bands were revealed when samples were incubated for 5–7 min. Samples were then incubated for 10 min with an equal volume of dextran-coated charcoal (0.25 g of acid-washed charcoal, 0.025 g of dextran T70 in 50 ml of PL buffer). The charcoal was then pelleted in a microcentrifuge and the supernatant containing the photoaffilated proteins resolved by SDS-PAGE using 10% acrylamide gels at 25 mA/gel in a Mighty Small® apparatus ( Hoefer Scientific). Proteins were transferred from the gels to nitrocellulose at 120 V for 1 h and the nitrocellulose subsequently dried in an oven for 20 min at 80 °C. The nitrocellulose was sprayed with Enhance® (Du Pont-New England Nuclear) and the photoaffiliated proteins visualized after exposure to x-ray film for 3–4 days. Radiolabeled protein band intensity on the x-ray film, which was used as an indicator of the relative amount of the protein present in the sample, was quantitated by laser densitometry (Waxman et al., 1988). Densitometric quantitations of the radiolabeled protein bands were found to be linear for at least a range of 0.25–4 times the standard load of cytosolic protein from an untreated adult male rat.

A competition binding studies were performed using this same general procedure except that unlabeled competitor (dissolved in ethanol) was added to each tube at the desired concentration and the solvent evaporated under a stream of nitrogen before adding the other constituents.

Density Gradient Centrifugation—Continuous 5–20% (w/v) sucrose gradients were prepared in PL buffer. Photoaffinity labeled cytosol preparations (200–400 µl at 10 mg/ml) were layered onto the top of the gradients (5 ml) and the samples centrifuged at 100,000 × g for 2.5 h. Bovine serum albumin (4.6 S) and rabbit IgG (7.1 S) were used as sedimentation markers. The gradient was then fractionated into 200-µl aliquots, 150 µl of which was used to measure radioactivity by scintillation counting and the remaining sample analyzed by SDS-PAGE and fluorography, as described above, to localize the individual radiolabeled proteins within the gradient.

RESULTS

Identification of SBPs by [3H]Methyltrienolone Photoaffinity Labeling—Photoaffinity labeling of cytosol from adult male rat liver with [3H]methyltrienolone revealed three major binding proteins having apparent molecular weights of 27 kDa (SBP27), 34 kDa (SBP34), and 45 kDa (SBP45) (Fig. 1, lane 1). In contrast, in experiments using cytosol from female rats, although the proteins of 27 and 45 kDa were also observed, no 34-kDa protein was detectable. Moreover, a methyltrienolone-binding protein of 31 kDa (SBP31) that was undetectable in the male was observed as a major binding protein in the female cytosol (Fig. 1, lane 8). These same binding patterns were obtained at methyltrienolone concentrations ranging from 10 to 150 nM, (data not shown). In control experiments, photoaffinity labeling of rat serum preparations revealed a single major methyltrienolone-binding protein of M, ~70 kDa, indicating that the cytosolic SBPs are not serum-derived contaminants (data not shown). The 70-kDa serum protein, which was also detected at a low (albeit variable) level in the cytosol preparations, is presumed to be albumin. No radioactive bands at these molecular weights were detected when microsomal preparations isolated from rat liver were photoaffiliated with [3H]methyltrienolone, thus demonstrating that the photoaffiliated SBPs are cytosolic proteins, and not contaminants of the microsomal fraction. Further support for the specific nature of SBP photoaffiliation was provided by the
proteins were photoaffinity labeled with $[^3H]$methyltrienolone, sub-
androgen groups: lane I, unaltered adult male rat (UT); lane 2, castrated male
lanes contain liver cytosol prepared from the following rat treatment
fluorography as described under "Materials and Methods." Individual
3 weeks after surgery (CX); lane 3, castrated male that received daily
androgen injections for 1 week (0.625 mg/kg
male; lane 5, hypophysectomized male; lane 6, hypophysectomized
male that received daily
androgen osmotic minipump
(0.63 µg/h for 7 days). These results demonstrate
significant reduction of incorporated radioactivity when the
photolabeling was carried out in the presence of a large excess
methyltrienolone (data not shown).

Density Gradient Centrifugation—Centrifugation of photo-
labelled liver cytosolic proteins through sucrose gradients re-
vealed that the majority of binding activity migrated to $\sim 4$ S
in both male and female cytosols (Fig. 2A, fractions 5–7), with a
small shoulder of binding activity also detected at $\sim 8$ S (Fig.
2A, fractions 13 and 14). SDS-PAGE and autoradiography of individual
fractions on SDS-PAGE revealed that the
SBP27 and SBP34 from male rat cytosol migrated to $\sim 4$ S
while SBP45 migrated to $\sim 8$ S (Fig. 2B). In female rat
preparations, SBP27 and SBP31 migrated to $\sim 4$ S and SBP45
migrated to $\sim 8$ S (data not shown). These results demonstrate
that while a single protein is responsible for the majority of
$[^3H]$methyltrienolone protein binding in the 8 S region of the gradient
while SBP45 migrated to $\sim 8$ S (data not shown).

Steroid Hormone Dependence of SBP34 and SBP31—The
differential expression of SBP34 and SBP31 in male and
female rats prompted an examination of the involvement of
gonadal hormones in the expression of these hepatic proteins.
Ablation of circulating androgen levels by castration of adult
male rats led to a progressive loss of SBP34 while having little
consistent effect on SBP27 and SBP45 (Fig. 1, lane 2, Fig.
3A). Treatment of the castrated rats with androgen reconstituted
near normal SBP34 levels (Fig. 1, lane 3; Fig. 3A). High level
expression of SBP34 was also obtained in ovariec-
tomized female rats treated with androgen (Fig. 3D). Conversely,
SBP34 levels in unaltered male rats were markedly suppressed
following treatment with the estrogen diethylstilbestrol (Fig.
1, lane 7; Fig. 3A). Thus SBP34 expression is positively
regulated by androgen and can be suppressed by estrogen.

Expression of the female-specific SBP31 was suppressed by
androgen, as was demonstrated by the loss of this protein in
ovariectomized female rats administered androgen for 1 week
(Fig. 3C) and by its partial expression in castrated males
($\sim 20$% of normal female level 9 weeks after castration) (Fig.
3B). Ovariectomy alone, however, did not result in significant

loss of SBP31 (Fig. 3C), indicating that estrogen does not
play an obligatory role in the expression of SBP31 in female
rats. These experiments demonstrate that the sex specificities
of SBP34 and SBP31 are due, at least in part, to their
differential regulation by steroid hormones. In contrast,
SBP27 and SBP45 were not significantly affected by these
hormones (Fig. 1 and data not shown) and therefore are
expressed in both sexes.

Regulation of SBP34 and SBP31 by Pituitary Hormones—
Since many of the effects of androgens and estrogens on
hepatic protein expression are mediated by the hypothalamic-
pituitary axis (Gustafsson et al., 1983), the involvement of
pituitary hormones, particularly growth hormone, in the ste-
roid hormone dependence of SBP34 and SBP31 was exam-
ined.

SBP34 is dependent upon pituitary factors for full expres-
sion as evidenced by its depression in male rats following
hypophysectomy (Fig. 1, lane 4; Fig. 4A, lane 3). The magni-
tude of loss of SBP34 following hypophysectomy was variable
among individual rats from several experiments ($n = 10$) with
an average loss of $\sim 50$%. Growth hormone does not appear
to contribute to SBP34 expression since twice daily injections
of the hormone, intended to mimic the pulsatile plasma
growth hormone pattern characteristic of male rats, had no
effect on SBP34 levels (Fig. 4A, lane 4). In contrast, daily
androgen administration to hypophysectomized male rats re-

\begin{figure}
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\caption{Fig. 1. Visualization of SBPs by photoaffinity labeling of rat liver cytosol with $[^3H]$methyltrienolone. Shown is the response of SBP31 and SBP34 to hormonal alterations. Cytosolic proteins were photoaffinity labeled with $[^3H]$methyltrienolone, subject to SDS-PAGE, transferred to nitrocellulose, and visualized by fluorography as described under "Materials and Methods." Individual lanes contain liver cytosol prepared from the following rat treatment groups: lane 1, unaltered adult male rat (UT); lane 2, castrated male 3 weeks after surgery (CX); lane 3, castrated male that received daily androgen (AND) injections for 1 week beginning 5 weeks after surgery (0.625 mg/kg methyltrienolone); lane 4, hypophysectomized (HX) male; lane 5, hypophysectomized male implanted with a growth hormone osmotic minipump (0.63 µg/h for 7 days); lane 6, hypophysectomized male that received daily androgen injections for 1 week (0.63 µg/kg methyltrienolone); lane 7, unaltered male that was infused with synthetic estrogen (EST) for 1 week (1.0 µg/h diethylstilbestrol); lane 8, unaltered female; lane 9, hypophysectomized female; lane 10, hypophysectomized female implanted with a growth hormone minipump (0.63 µg/h for 7 days).
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\caption{Fig. 2. Sucrose gradient fractionation of cytosolic proteins photolabeled with $[^3H]$methyltrienolone. A, radioactivity associated with individual gradient fractions from both male (C) and female (○) rat liver cytosol. Gradient markers are bovine serum albumin (4.6 S) and rabbit IgG (7.1 S). B, densitometric quantitation of individual photoaffinity labeled proteins from male rat cytosol present in selected gradient fractions. The individual proteins that constituted the binding in the 4 S and 8 S regions of the gradient in A were examined by subjecting individual fractions from the sucrose gradient to SDS-PAGE, transferring the separated proteins to nitrocellulose, subjecting the nitrocellulose to fluorography, and finally measuring band intensities on the resultant x-ray film by laser densitometry. The locations of the individual SBP proteins in the gradient are indicated by the following: SBP27 (○), SBP34 (△), SBP45 (▲). In addition, a significant percentage of the radioactivity associated with fractions 1–9 could be attributed to low molecular weight $[^3H]$methyltrienolone-binding material that migrated with the buffer front during electrophoresis (○). Parallel analysis of female rat liver cytosol revealed that SBP31 migrated in the 4 S region (data not shown).
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FIG. 3. Influence of gonadal hormones on SBP levels. A and B, effects of castration and steroid hormone supplementation on the expression of SBP34 (A) and SBP31 (B) in male rat liver cytosol. Relative SBP levels were assayed following photoaffinity labeling with [3H]methyltrienolone as described in Fig. 1. Lane 1, unaltered male rats (UT); lane 2, rats killed 3 weeks following castration; lane 3, rats killed 9 weeks following castration; lane 4, rats killed 3 weeks following castration and administered androgen (0.625 mg/kg methyltrienolone) daily for 1 week prior to killing; lane 5, unaltered rats that received estrogen infusion (1.0 μg/h diethylstilbesterol) for 1 week prior to death. Brackets, S.E. response of three to four rats per treatment group.

FIG. 4. Response of SBP34 (A) and SBP31 (B) to hypophysectomy and growth hormone replacement in male and female rats. Relative SBP protein levels were determined following photoaffinity labeling as described in Fig. 1. Lane 1, untreated, sham-operated male rats (UT); lane 2, sham-operated male rats implanted with a growth hormone minipump (0.63 μg/h for 7 days); lane 3, hypophysectomized male rats; lane 4, hypophysectomized male rats that received twice daily human growth hormone injections (30 μg/week) for 7 days (GHII); lane 5, hypophysectomized male rats implanted with a growth hormone minipump (0.63 μg/h for 7 days); lane 6, hypophysectomized male rats that received daily androgen injections for 1 week (0.625 mg/kg methyltrienolone); lane 7, untreated, sham-operated female rats; lane 8, hypophysectomized female rats; lane 9, hypophysectomized female rats implanted with a growth hormone minipump (0.63 μg/h for 7 days). Brackets, S.E. response of four rats per treatment group. Dashed line, normal level of SBP31 in untreated adult female rats (B and C) or of SBP34 in untreated adult male rats (D). Ovariectomy alone (O-day androgen administration data points) is shown to have no suppressive effect on normal female SBP31 levels and no stimulatory effect on SBP34 levels.

resulted in a substantial restoration of SBP34 after 1 week (Fig. 1, lane 6; Fig. 4A, lane 6) and fully restored this protein after 2 weeks (data not shown). Similar results were obtained when endogenous androgen production was stimulated in the hypophysectomized male rats by human chorionic gonadotropin (150 IU/kg subcutaneously, daily for 7 days). Thus, in contrast to other androgen-dependent rat hepatic proteins (Gustafsson et al., 1983), SBP34 can be stimulated by androgen by a mechanism that does not require the participation of other pituitary-dependent hormones. Moreover, the loss of SBP34 following hypophysectomy seems to be due to the loss of gonadotropins and ultimately the depletion of circulating androgen.

While androgen, and not intermittent growth hormone, seems to be the pituitary-dependent factor that contributes positively to the expression of SBP34 in male rats, continuous growth hormone exposure can exert a suppressive influence on SBP34 levels. Continuous plasma growth hormone levels, characteristic of adult female rats and achieved in our experiments by use of an osmotic minipump (see “Materials and Methods”), severely suppressed this protein in both hypophysectomized and unaltered male rats (Fig. 1, lane 5; Fig. 4A, lanes 2 and 5).

Female-specific SBP31 was markedly suppressed by hypophysectomy, indicating that it also requires pituitary-dependent factors for full expression (Fig. 1, lane 9; Fig. 4B, lane 8). Continuous infusion of growth hormone to hypophysectomized female rats led to a small increase in the level of this protein, as was also observed in unaltered and hypophysec-
tomized male rats treated continuously with growth hormone under the same conditions (Fig. 1, lanes 5 and 10; Fig. 4B, lanes 2, 5, and 9). This finding suggests that other pituitary-dependent hormones may be required to act in concert with growth hormone to achieve full expression of SBP31.

**Modulation of SBP34 and SBP31 by Drug Treatment.**—The expression of many hepatic enzymes involved in sterol metabolism can be modulated following exposure to drugs or other foreign compounds. If SBP34 and SBP31 function in concert with sterol-metabolizing enzymes, then their expression might also be influenced by such drugs. Therefore, the effect on SBP levels of several drugs that are known inducers of hepatic steroid-metabolizing enzymes was evaluated. The male-specific SBP34 was not induced by phenobarbital, dexamethasone, or clofibrate in either sex; rather, this protein was variably suppressed by these compounds, with dexamethasone effecting complete suppression of this protein in males (Fig. 3A). Analysis of serum testosterone levels in the drug-treated rats revealed that dexamethasone also eliminated circulating levels of this hormone (Table I). In view of the androgen dependence of SBP34 (Fig. 3A), it seems likely that the loss of circulating androgen in these animals is responsible for the dexamethasone-induced loss of SBP34. The female-specific SBP31 was not elevated by any of the drugs tested, although it was partially suppressed in females by phenobarbital (Fig. 3B). Thus, none of the compounds examined induced expression of either SBP34 or SBP31 in liver cytosol.

**Ligand-binding Specificity of SBPs.**—The ligand-binding specificities of SBP34 and SBP31 were examined in competition experiments using both anabolic and catabolic sterol metabolites. Included were cholesterol, triamcinolone acetonide (a synthetic glucocorticoid) and progesterone, the gonadal steroids (testosterone, estradiol) and some of their metabolites (5a-dihydrotestosterone, 5a-androstan-3α,17β-diol), and both primary bile acids (cholic acid, chenodeoxycholic acid) and secondary bile acids (deoxycholic acid, lithocholic acid).

Female-specific SBP31 exhibited no significant binding affinity toward cholesterol and triamcinolone acetonide as evidenced by the inability of these steroids to compete with [3H]methyltrienolone when included in the photolabeling mixture at 12 μM, corresponding to a 100-fold molar excess over methyltrienolone (Fig. 6A). In contrast, progesterone was 75% effective at reducing the labeling of SBP31 by [3H]methyltrienolone under comparable incubation conditions (Fig. 6A). The sex steroids (testosterone, 5α-dihydrotestosterone, and estradiol) were moderately effective competitors, with ~50% inhibition of [3H]methyltrienolone incorporation at 12 μM, while the secondary testosterone metabolite 5α-

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**Table 1**

| Treatment       | Serum testosterone* (ng/ml) |
|-----------------|----------------------------|
| Untreated       | 1.4 ± 0.5                  |
| Phenobarbital   | 1.8 ± 1.0                  |
| Dexamethasone   | <0.1                       |
| Clofibrate      | 1.3 ± 1.0                  |

* Mean ± S.E. (n = 3). Values determined by radioimmunoassay as described previously (LeBlanc and Waxman, 1988).

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**Fig. 5.** Response of SBP34 (A) and SBP31 (B) to drug treatments in male and female rats. Drugs were administered as described under "Materials and Methods" and individual SBPs quantitated using photoaffinity labeling as described in Fig. 1. UT, untreated rat; PB, phenobarbital-treated rat; DEX, dexamethasone-treated rat; CF, clofibrate-treated rat. Brackets, S.E. response from three to four rats per treatment group.

**Fig. 6.** Competition by steroids for SBP31 photoaffinity labeling by [3H]methyltrienolone. Cytosols from adult female rat liver were incubated with [3H]methyltrienolone in the presence of 1.2, 6.0, or 12 μM unlabeled steroid compounds. Binding of the steroid compounds to SBP31 was revealed by the reduced labeling of the SRP by [3H]methyltrienolone. The relative level of photoaffinity labeling was then quantitated as described in Fig. 1. Ligands used in each experiment were as follows: A, triamcinolone acetonide (TA, ○), cholesterol (Chol, ●), progesterone (Prog, △); B, 5α-androstan-3α,17β-diol (Adiol, ○), testosterone (Test, ○), 5α-dihydrotestosterone (DHT, Δ), estradiol (E2, △). C, cholic acid (Cholic, O), chenodeoxycholic acid (Chen, ●), deoxycholic acid (DOC, Δ), lithocholic acid (Lith, ●).
unlabeled steroid compounds were used as described in Fig. 6. Labeling by [3H]methyltrienolone. of 6 acids cholic acid and chenodeoxycholic acid, with -80% inhibition of [3H]methyltrienolone binding at a concentration However, chenodeoxycholic acid, and to a somewhat lesser androstan-3a,17β-dial was ineffective (Fig. 6B). SBP31 did not exhibit significant binding affinity for the primary bile acid cholic acid or its metabolite deoxycholic acid (Fig. 6C). However, chenodeoxycholic acid, and to a somewhat lesser extent its metabolite lithocholic acid, inhibited [3H]methyltrienolone incorporation in the low micromolar range (Fig. 6C). Chenodeoxycholic acid, the more effective competitor, is found in female rat liver cytosol at a concentration of ~40 µM, while lithocholic acid is present at a somewhat lower level (Kurtz et al., 1982). This suggests that these bile acids might serve as physiologically significant ligands for SBP31. Male-specific SBP34 exhibited little binding affinity toward cholesterol, triamcinolone acetonide, progesterone, testosterone, androstenedione, and its derivatives, and estradiol (Fig. 7, A and B). However, this protein did effectively bind the primary bile acids cholic acid and chenodeoxycholic acid, with ~80% inhibition of [3H]methyltrienolone binding at a concentration of 6 µM (Fig. 7C). SBP34 bound the secondary bile acid deoxycholic acid with similar affinity as the primary bile acids (Fig. 7C). Lastly, SBP34 also bound lithocholic acid, but less effectively than the other bile acids, particularly at the lower concentrations tested. These results suggest that SBP34 is also a major binder of bile acids. However, SBP34 differs from SBP31 in its much higher affinity for cholic acid and its lower affinity for progesterone and the gonadal steroids.

**DISCUSSION**

The present study establishes that male and female rats each express a unique hepatic protein with the properties of a cytosolic bile acid-binding protein. These sex-dependent proteins, which are of similar size and have similar sedimentation characteristics, are differentially regulated by steroid and peptide hormones. It is this differential hormone regulation that confers the sex-specific expression that is characteristic of these hepatic proteins.

The expression of the male-specific SBP34 appears somewhat analogous to that of a male-specific liver microsomal steroid 16α-hydroxylase cytochrome P-450 enzyme, designated P-450<sub>K</sub> (P-450 gene product IIC11). Like SBP34, P-450<sub>K</sub> is dependent upon androgen for full expression (Waxman et al., 1985), and is also suppressed by several drugs that are known to induce other hepatic proteins (Dannan et al., 1983; Yeowell et al., 1987). However, the regulatory mechanisms that dictate the male expression of these two hepatic proteins apparently differ. P-450<sub>K</sub> expression is largely dependent upon pulsatile growth hormone secretions, and the role of androgen in the expression of this protein is believed to reflect the stimulation by androgen of a pulsatile growth hormone secretory profile (Morgan et al., 1985; Kato et al., 1986; Jansson and Frohman, 1987). SBP34 expression, however, is unresponsive to pulsatile growth hormone and, in addition, its responsiveness to androgen does not require an intact pituitary gland. Both proteins, however, are suppressed by estrogen and by continuous plasma growth hormone levels (this study and Morgan et al., 1989).

Like SBP34, SBP31 is also under multiple hormonal regulatory controls. The expression of this female-specific protein is suppressed by androgen and appears to be positively influenced by growth hormone and perhaps by estrogen as well. The pattern of regulation of this protein by hormones is similar to that of the female-predominant microsomal enzyme steroid 5α-reductase, whose expression is partly derepressed by castration of adult male rats, and can be stimulated (albeit to a fuller extent that SBP31) by continuous growth hormone treatment of unaltered males or hypophysectomized male and female rats (Mode et al., 1981; Waxman et al., 1989a).

Other investigators have identified steroid-binding activity associated with proteins that have sucrose gradient sedimentation characteristics similar to the steroid-binding proteins identified in the present study (Roy et al., 1983; Powell-Jones et al., 1980). Roy et al. (1983) characterized an androgen- and estrogen-binding activity from rat liver cytosol that sediments at ~3.5 S and was proposed to be a receptor for 5α-dihydrotestosterone. The protein(s) responsible for this binding activity exhibited high affinity toward testosterone, 5α-dihydrotestosterone, and 17β-estradiol and appeared to be expressed only in male rats.

Similarly, Powell-Jones et al. (1980) reported a male-specific protein with high binding affinity for both androgens and estrogens. This binding activity was greatly reduced in cytosol from hypophysectomized male rats, but was elevated in cytosol from hypophysectomized female rats. Unfortunately, since these characterizations only reflect changes in total ligand-binding activity in a crude cell fraction, it is impossible to ascertain whether a single or multiple proteins contribute to the observed ligand-binding profiles. Nonetheless, competition binding experiments performed in the present study with SBP34 indicate that testosterone, 5α-dihydrotestosterone and estradiol are relatively poor ligands for this protein, suggesting that SBP34 is probably not responsible for the male-specific binding activity characterized in the earlier studies.

SBP34 expression in male rat liver was suppressed by the estrogen diethylstilbestrol, while the antiandrogen hydroxyflutamide (SCH 16423) had little effect on the level of SBP34 expression. These observations are similar to those made

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2 G. A. LeBlanc and D. J. Waxman, unpublished data.
with a male estrogen-binding protein designated MEB (Eagon et al., 1989). Other similarities between SBP34 and MEB are their male specificity and their decreases following castration and hypophysectomy. However, these proteins are apparently distinct insofar as MEB does not bind methylthrienolone (Eagon et al., 1989) and has a high binding affinity toward estradiol (Eagon et al., 1989) that is apparently not shared by SBP34.

Recently, Demyan et al. (1989) purified a 31-kDa cytosolic androgen-binding protein, designated CAB, that is expressed in male but not female rat liver. Earlier studies by the same investigators revealed the existence of a female-specific 29-kDa cytosolic androgen-binding protein in rat liver (Sarkar et al., 1987). Since both proteins were visualized using the [3H]methylthrienolone photoaffinity labeling techniques employed in the present study, it is concluded that they are, in fact, the same as the male-specific SBP34 and the female-specific SBP31 described in the present study and that the apparent differences in size in the two studies (CAB at 31 kDa versus SBP34 at 34 kDa; female-specific 29 kDa versus SBP31 at 31 kDa) reflect interlaboratory differences in the calibration of the SDS-PAGE systems rather than true differences in molecular mass. The physiological significance of these proteins has not been established. However, the male-specific CAB protein (i.e. SBP34) appears to be coregulated with α2u-globulin (Sarkar et al., 1987), a major urinary protein synthesized in male but not female rat liver (Roy et al., 1983). The CAB protein has been proposed to be a mediator of the androgen-dependent expression of α2u-globulin (Sarkar et al., 1987) and was suggested to be related to the androgen-binding domain of the androgen receptor (Demyan et al., 1989). The present demonstration of a significantly higher binding affinity of this male-specific protein toward bile acids as compared to androgens suggests, however, that it is unlikely to carry out androgen-dependent functions in the hepatocyte.

Competition binding experiments revealed that SBP34 and SBP31 both bind bile acids with comparatively high affinity. This observation, in the context of the high levels of bile acids found in the liver, strongly suggests that bile acids are the principal endogenous ligands for these proteins. The two sex-specific SBPs do differ, however, in their relative affinities for individual bile acids. The male-specific SBP34 was capable of binding both primary bile acids and their metabolites with high affinity, but exhibited no appreciable binding toward the secondary bile acids. SBP31 both bind bile acids with comparatively high affinity, but exhibited no appreciable binding toward the secondary bile acids (Kurtz et al., 1982). Furthermore, sex differences have been observed for a number of enzymes that carry out bile acid metabolism, including those active in sulfate conjugation (Barnes et al., 1979), hydroxylation (Yousef et al., 1973) and oxidoreduction (Björkhem et al., 1973). Thus, sex specificity with regard to ligand carrier proteins is not unexpected. Further investigation is required to address critical questions regarding the functions of these proteins. This includes a more thorough evaluation of the ligand specificities of these proteins, the extent to which they might confer protection from bile acid toxicity, and their potential roles in the metabolism of bile acids or in mediating their biological actions, such as the feedback inhibition of cholesterol 7α-hydroxylase, a key enzyme of the bile acid biosynthetic pathway (Miyat and Mitropoulos, 1977). Further characterization of these proteins may ultimately lead to a fuller understanding of their activities as well as the factors that contribute to pathological conditions related to bile acid transport and metabolism (Borgström et al., 1985; Björkhem, 1985).

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