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Photoaffinity labeling identification of thyroid hormone-regulated glucocorticoid-binding peptides in rat liver endoplasmic reticulum: an oligomeric protein with high affinity for 16β-hydroxylated stanozolol

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Abstract

Steroid-binding proteins unrelated to the classical nuclear receptors have been proposed to play a role in non-genomic actions of the17α-alkylated testosterone derivative (17α-AA) stanozolol (ST). We have previously reported that male rat liver endoplasmic reticulum contains two steroid-binding sites associated with high molecular mass oligomeric proteins: (1) the ST-binding protein (STBP); and (2) the low-affinity glucocorticoid-binding protein (LAGS). To further explore the role of LAGS on the mechanism of action of ST, we have now studied: (1) the interaction of ST and its hydroxylated metabolites with solubilized LAGS and the cytosolic glucocorticoid receptor (GR); and (2) the effects of hormones on the capability of STBP to bind ST. We found that, unlike 17α-methyltestosterone, neither ST nor its hydroxylated metabolites bind to GR. However, the 16β-hydroxylation of ST significantly increases the capability of LAGS to bind ST. Interestingly, 3′-hydroxylation of ST abrogates the capability of LAGS to bind ST. ST ($k_i = 30$ nM) and 16β-hydroxystanozolol ($k_i = 13$ nM) bind with high affinity to LAGS, and are capable of accelerating the rate of dissociation of previously bound dexamethasone from the LAGS. STBP and LAGS are strongly induced by ethinylestradiol. However, unlike STBP, LAGS is regulated by thyroid hormones and growth hormone, which proves that these steroid-binding activities are associated with different binding sites. These findings seem to suggest a novel mechanism for ST whereby membrane-associated glucocorticoid-binding activity is targeted by the 16β-hydroxylated metabolite of ST. ST and its 16β-hydroxylated metabolite modulate glucocorticoid activity in the liver through negative allosteric modulation of LAGS, with the result of this interaction an effective increase in classical GR-signaling by increasing glucocorticoid availability to the cytosolic GR.

Keywords: Membrane steroid receptors; LAGS; Glucocorticoids; Liver; Anabolic steroids

1. Introduction

The liver is an important target for 17α-alkylated testosterone derivatives (17α-AA) [1–4]. Many pathological conditions associated with prolonged use of 17α-AA emanate from the adverse effects of these steroids on hepatic gene expression. Their genomic effects are thought to occur predominantly through binding to the androgen receptor (AR), a member of the nuclear receptor superfamily that functions as a ligand-activated transcription factor. There is also indirect evidence that the effects of androgens on tissues may be mediated by antagonizing the action of glucocorticoids, by direct competition for the cytosolic glucocorticoid receptor (GR) [1–4]. This seems plausible, as there exists a significant degree of homology in the ligand-binding domain of the AR with other members of the steroid hormone receptor

Abbreviations: 17α-AA, 17α-alkylated androgen derivatives; AR, androgen receptor; B_{max}, maximal binding; D_2O, deuterium oxide; DA, danazol; DCC, dextranT70-coated charcoal; DTT, dithiothreitol; DEX, dexamethasone; 16β-OHST, 16β-hydroxystanozolol; 3-OHST, 3′-hydroxystanozolol; GR, glucocorticoid receptor; $GABA_AR$, $GABA_A$ receptor; LAGS, low-affinity glucocorticoid-binding site; MMI, 2-mercapto-1-methylimidazole; mGR, membrane-glucocorticoid receptor; mPBP, membrane-progesterone-binding protein; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; ST, stanozolol; STBP, stanozolol-binding protein; TMMDSI buffer, 50 mM Tris–HCl, 10 mM sodium molybdate, 5 mM magnesium chloride, 2 mM dithiothreitol, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.001 mM leupeptine, 0.01 mM aprotinine, and 1 μg/ml soybean trypsin inhibitor, pH 7.5; V_0, void volume; V_t, total liquid volume; TX, hypothyroid

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superfamily [5]. In addition, androgens exert non-genomic effects independent of gene transcription. Typically, their non-genomic effects are initiated at the plasma membrane and result in the regulation of membrane protein-mediated intracellular signaling pathways [5].

Interestingly, androgens can show very diverse profiles of biological actions. However, the molecular basis for this variety of biological actions is not well understood. Recently, it has been suggested that steroid-specific differences in gene transcription profiles due to AR activation could contribute to differences in biological actions of testosterone and 17α-AA [6]. There is also increasing evidence that these differences may be mediated by the actions of 17α-AA on tissues through mechanisms independent of AR. For example, 17α-AA stanozolol (ST) (but not testosterone or its esters) increases the concentrations of several plasma glycoproteins synthesized in the liver [1], triggers immediate-early gene expression by a mechanism independent of the AR [7], stimulates collagen synthesis in fibroblasts through a mechanism mediated by transforming growth factor-β1 [8], and induces allosteric regulation of established and putative membrane receptors, e.g. the $GABA_A$ receptor ($GABA_AR$) [9] and the low-affinity glucocorticoid-binding site (LAGS) [10]. Furthermore, structural modifications make 17α-AA more resistant than testosterone to hepatic metabolism, which may contribute to generate steroid-specific differences in biological actions associated with significant hepatic toxicity [1,2,4]. Although ST is more resistant than testosterone to hepatic metabolism, it is also metabolized in rats and man to a large extent [11], with two major metabolites identified: 3′-hydroxystanozolol (3-OHST) and 16β-hydroxystanozolol (16-OHST). Thus, the generation of active metabolites, at least in part, may also explain differences in biological effects of testosterone and ST.

We have demonstrated that the male rat liver endoplasmic reticulum contains a high-affinity ST-binding protein (K_d = 30 nM) (STBP) [10]. This is an oligomeric protein with constituent peptides are under hormonal regulation, and can be saturated at the dose of ST used for treatment of various diseases [12] and abused by athletes [1–4]. We have hypothesized that ST, through its interaction with STBP, exerts a negative allosteric modulation of glucocorticoid-binding activity carried out by LAGS. LAGS has a molecular mass of at least 135 kDa, is an endoplasmic reticulum-associated glucocorticoid-binding protein, is capable of interacting with steroids with a lower affinity than that exhibited by the respective nuclear receptors for these hormones, and does not bind 17β-estradiol or testosterone [13,14]. Thus far, the evidence suggests that steroids LAGS is capable of binding have powerful effects on the liver [13], that ST can interact and modulate LAGS activity under physiological and pharmacological conditions [10,12], that LAGS can modulate glucocorticoid action in vivo [15], that LAGS does not mediate glucocorticoid-dependent induction of CYP3A2 in rat liver [16], and that LAGS is under physiological regulation by glucocorticoids, thyroid hormones, growth hormone, and Vitamin A [16–18].

In the present work, we have further characterized the hydrodynamic properties of LAGS. Photoaffinity labeling, a powerful tool for the identification and characterization of steroid-specific mediators of biological and pharmacological phenomena [19], has been used to identify thyroid hormone-regulated glucocorticoid-binding peptides in LAGS. We have found that 16β-OHST is both a high-affinity-binding ligand for and an allosteric inhibitor of this endoplasmic reticulum-associated glucocorticoid-binding protein. Interestingly, neither ST nor its hydroxylated metabolites interact with cytosolic GR, which makes unlikely the possibility that this receptor mediates direct ST actions on the liver. These findings seem to suggest a novel mechanism by which ST may modulate glucocorticoid activity in the liver through the negative allosteric modulation of membrane-associated glucocorticoid-binding proteins. The result of this interaction, in contrast with the antiglucocorticoid hypothesis, would be the effective increase of glucocorticoid availability to the cytosolic GR.

2. Materials and methods

2.1. Reagents

[^{3}H]ST (25 Ci/mmol) was synthesized by SibTech Inc. (Elmsford, NY). 16β-Hydroxystanozolol and 3′-hydroxystanozolol were purchased from Cerilliant (Austin, TX). [^{3}H]DEX (45 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Ready Safe scintillation cocktail was purchased from Beckman Instruments Inc. (Palo Alto, CA). Human recombinant GH (hGH) was kindly provided by Pharmacia (Spain). Unless otherwise indicated, the rest of the products cited in this work were purchased from the Sigma (St. Louis, MO).

2.2. Animals

Adult (2–3-month-old) male Sprague-Dawley rats were used throughout these experiments. Hypothyroidism (TX) was induced in adult male rats by administering 0.05% 2-mercapto-1-methylimidazole (MMI) in the drinking water for 21 days [20]. Thyroid hormones were dissolved in a small volume of 0.01 N NaOH and were brought up to a series of concentrations with sterile saline; they were then administered as a single daily i.p. injection at the doses and on the days specified. Control animals received the same volume (0.25 ml) of the appropriate vehicle. Where indicated, hGH was administrated as a s.c. injection in sterile physiological saline at the stated doses, divided into three daily injections. All the animals were sacrificed by decapitation 24 h after the last injection, and their livers were quickly removed and washed in ice-cold saline. Animals were maintained and sacrificed in accordance with

institutional guidelines for the care and use of laboratory animals.

2.3. Preparation of cytosol and microsomes

All steps were carried out at 0–4 °C. Liver samples were homogenized in a Teflon-glass Potter-Elvehjem homogenizer (B. Braun, Melsungen, Germany) in TMMDSI buffer (millimolar): Tris–HCl, 50; sodium molybdate, 10; magnesium chloride, 5; dithiothreitol (DTT), 2; sucrose, 250; phenylmethylsulfonyl fluoride, 1; EDTA, 1; leupeptin, 0.001; aprotinin, 0.01; and 1 μg/ml soybean trypsin inhibitor, pH 7.5. Homogenates were centrifuged at 17,000 × *g* for 15 min. The supernatant was then centrifuged at 105,000 × *g* for 1 h, and the pellets (microsomes) resuspended in TMMDSI buffer and recentrifuged at 105,000 × *g*. The supernatant was centrifuged again at 105,000 × *g* for 1 h to obtain cytosol. The centrifuged pellet (microsomes) was resuspended in an appropriate volume of TMMDSI buffer to give a protein concentration of 2–3 mg/ml. The cytosol and microsomal suspensions were used for the binding assays.

2.4. Radioligand-binding assays

For GR measurement, aliquots of cytosol were incubated overnight at 0–4 °C in duplicate with increasing concentrations (from 0.5 to 50 nM, final concentration) of [^{3}H]DEX. For the LAGS activity, 200 μg of microsomal suspensions or solubilized proteins were incubated overnight at 0–4 °C in duplicate with increasing concentrations (from 5 to 500 nM, final concentration) of [^{3}H]DEX. Nonspecific binding for both GR and LAGS activities was measured at parallel incubations with a 200-fold excess of unlabeled DEX. At the end of the incubation period, a suspension of 200 μl of dextran T70-coated charcoal (DCC, 0.08–0.8%, final concentration) in TMMDSI buffer was added, and the samples were shaken, incubated for 10 min at 0–4 °C and centrifuged. At this DCC concentration, the nonspecific binding represented less than 10% of the total binding; higher concentrations of DCC did not yield greater effectiveness. Nonspecific binding showed an excellent linearity in the whole range of concentrations assayed. Aliquots of the supernatant were taken for radioactivity counting. In the control experiments, incubations were carried out in the presence of the vehicle alone (2% ethanol final concentration). No effect of ethanol (1–5%) was observed.

2.5. Subcellular fractionation of rat liver membranes

In order to examine the subcellular distribution of the [^{3}H]DEX-binding activity, the livers obtained from two adult male rats were perfused with ice-cold physiological saline, as previously described, and homogenized in four volumes of ice-cold TMMDSI buffer. All subsequent steps were performed at 4 °C. The homogenate was filtered through a nylon bolting cloth (pore size 50 μm), and the filtrate was centrifuged twice at 1000 × *g* for 10 min. The nucleus and plasma membrane-enriched fractions were obtained from this pellet [21], according to procedure, using a Percoll gradient [22]. The 1000 × *g* supernatant was fractionated, as described for rat liver [23]. The 1000 × *g* supernatant previously obtained was centrifuged at 10,000 × *g* for 15 min. Approximately half of this pellet was suspended in TMMDSI buffer (total volume 6 ml), and 1.5 ml of suspension was layered on 10 ml of iso-osmotic 40% Percoll solution ($\rho \approx 1.08\,g/cm^3$). This was then centrifuged at 60,000 × *g* (70.1Ti fixed-angle rotor, Beckman Instruments, Fullerton, CA) for 30 min. Thus, the three fraction—an upper half portion (peroxisome fraction, $\rho < 1.070\,g/cm^3$); a lower half portion (mitochondria-enriched fraction, $1.070 \leq \rho < 1.128\,g/cm^3$); and a portion adjacent to the bottom (lysosome-enriched fraction, $\rho \approx 1.123\,g/cm^3$)—were roughly separated. Each fraction was washed three times with TMMDSI by means of centrifugation at 16,000 × *g* for 20 min.

The 10,000 × *g* supernatant was centrifuged at 105,000 × *g* for 60 min, and the resultant supernatant recentrifuged under the same conditions and used as the cytosolic fraction. The first 105,000 × *g* pellet was used to obtain smooth- and rough-endoplasmic reticulum (SER and RER) in accordance with a modified Rostchild method [24]. Briefly, the pellet was resuspended in TMMDSI containing a sufficient amount of sucrose to give 0.15 M, final concentration, and 7.5 ml of this solution layered onto 2 ml-cushion of TMMDSI-1.3 M sucrose and centrifuged for 100 min at 320,000 × *g*. After this, the RER fraction could be recovered as a sediment at the bottom of the tube, whereas SER formed a band at the interface that, once diluted 1:3 with ice-cold TMMDSI was recovered by centrifugation at 105,000 × *g* for 60 min.

All fractions obtained above were flash frozen in liquid nitrogen and stored at −80 °C. [^{3}H]DEX-binding activity and the activity of the following marker proteins were determined in all fractions: NADPH cytochrome *c* reductase (endoplasmic reticulum), succinate dehydrogenase (mitochondria), 5′-nucleotidase (plasma membrane), and lactate dehydrogenase (cytosol) [24].

2.6. Solubilization of the LAGS

Unless otherwise indicated, extraction of proteins was accomplished by incubating microsomes (3 mg/ml) in the presence of either 7.5 mM CHAPS or 4.5 mM Triton X-100 in TMMDSI-0.1 M KCl buffer for 60 min by gentle agitation at 4 °C. Insoluble material was removed by ultracentrifugation at 105,000 × *g* × 60 min at 4 °C, and the resulting supernatant was filtered through a 0.22 μm Millex-GS sterile filter (Millipore, Bedford, MA). For some experiments peripheral membrane proteins were removed by pretreating microsomes with 0.75 M KCl–TMMDSI for 30 min at 0 °C, and the extracted microsomes collected by ultracentrifugation and solubilized with detergent, as described.

2.7. Phase separation of integral membrane proteins in Triton X-114 solution

Phase separation of integral membrane proteins in Triton X-114 solution was accomplished following Bordier [25]. Briefly, for the separation of proteins a cushion of 6% sucrose (w/v) in TMMDSI-0.03% Triton X-114 (500 μl) was placed at the bottom of a 1.5 ml conical Eppendorf microfuge tube. The clear sample was then overloaded on this sucrose cushion, and the tube incubated for 3 min at 30 °C. After centrifugation, the detergent phase was found as an oily droplet at the bottom of the tube. The upper aqueous phase was removed from the tube and made 0.3% with fresh Triton X-114. After dissolution of the surfactant at 0 °C, the mixture was again loaded onto the sucrose cushion previously used, incubated for 5 min at 30 °C, and centrifuged. At the end of the separation, the aqueous phase was rinsed with 1% Triton X-114 in a separate tube without a sucrose cushion, and the detergent phase discarded. After separation, Triton X-114 and buffer were added, respectively, to the aqueous and detergent phases in order to obtain equal volumes and approximately the same salt and surfactant content for both samples. Aliquots of the separated phases were analyzed by radioligand-binding assays.

2.8. Gel filtration chromatography of LAGS

Solubilized proteins were applied to a Sephacryl S-300 column (50 cm × 1.5 cm, Pharmacia LKB Biotechnology Inc.). The column was balanced with TMMDSI and either 7.5 mM CHAPS or 4.5 mM Triton X-100. The flow rate of the column was maintained at 18 ml/h, and 1 ml fractions were collected to measure specific [^{3}H]DEX-binding activity, by the method described above, and to determine protein concentration. The gel filtration column was calibrated using proteins of known Stokes radius under conditions identical to those used for LAGS. The following marker proteins were employed: carbonic anhydrase (1.60 nm); bovine serum albumin (BSA) (3.55 nm); alcohol dehydrogenase (4.75 nm); and apoferritin (6.10 nm). Blue dextran 2000 and $K_3Fe(SCN)_6$ were used to determine the void volume (V_0) and the total liquid volume (V_t) of the column, respectively. Retention of proteins in the matrix was evaluated by the partition quotient (K_{AV}), defined as $K_{AV} = (V_e - V_0)/(V_t - V_0)$, V_e being the elution volume of the protein. Calibration curves were constructed by plotting the Stokes radii versus $1 - K_{AV}$, in accordance with Ackers' method [26].

2.9. Sucrose density gradient ultracentrifugation

The sedimentation quotient ($s_{20,w}$) and the partial specific volume of LAGS-detergent complexes were determined by centrifugation through sucrose density gradients in H_2O or D_2O [27]. Linear gradients (4 ml) of 4–20% (w/v) sucrose in TMMDSI containing 4.5 mM Triton X-100 or 18–34% (w/v) containing 7.5 mM CHAPS were prepared in H_2O or D_2O. The gradients were allowed to cool at 4 °C, and 100 μl (about 2 mg of protein) of incubated samples were layered on top of the gradients. On parallel gradients the following standard proteins were sedimented in the same run: cytochrome *c* ($s_{20,w}$ = 1.17); carbonic anhydrase ($s_{20,w}$ = 2.75); BSA ($s_{20,w}$ = 4.6); alcohol dehydrogenase ($s_{20,w}$ = 7.4); and β-amylase ($s_{20,w}$ = 8.9). Centrifugation was carried out at 4 °C for 16 h with a Beckman SW60 rotor at 225,000 × *g* for sucrose/H_2O gradients, or 325,000 × *g* for sucrose/D_2O gradients. Following centrifugation, twenty-five 150 μl-fractions were collected from the bottom of the tube. [^{3}H]DEX-binding activity, protein concentration, and sucrose density in the different fractions were determined. Migration of standard proteins was measured by determining protein concentration in each of the fractions. The refractive index of each fraction was checked to assure the linearity of the gradient. The density of each fraction was determined by direct weighing of fixed volumes of sample. The $s_{20,w}$ and the partial specific volume of DEX-detergent complexes were calculated in accordance with Clarke [28], assuming that the partial specific volume of the protein component is 0.74 cm^3/g and that the same amount of detergent is bound to the protein in both H_2O and D_2O. The molecular masses of the DEX–detergent complexes were calculated from their Stokes radius, partial specific volume, and $s_{20,w}$ values using Svedberg's equation [29]. Data are given as means ± S.D.

2.10. Photoaffinity labeling of the LAGS

[^{3}H]DEX (300 nM) was incubated in the dark with 200 μg of microsomal membranes or CHAPS solubilized proteins in TMMDSI buffer overnight at 4 °C in the absence or presence of a 200-fold higher concentration of non-labeled ligand. Samples of 50 μl were removed in triplicate to measure reversible binding before irradiation. The incubation mixture was then transferred to wells of 24 well-plates (i.d. 15 mm) and irradiated for 22 min at 0 °C with a 254 UV lamp (Stratagene, La Jolla, CA) at a distance of 100 mm above plate. Nonspecific binding was defined with 60 μM DEX. Photolabeled samples were concentrated by centrifugation with Centriplus YM (Millipore, Bedford, MA). Samples were diluted with an equal volume of sample buffer (60 mM Tris–HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 2% (w/v) SDS, 100 mM DTT, and 0.001% (w/v) bromophenol blue), then heated at 95 °C for 5 min, and analyzed by SDS-PAGE, or quick-frozen with liquid nitrogen and stored at −70 °C until needed.

2.11. SDS-PAGE of photolabeled LAGS

SDS-PAGE was carried out on polyacrylamide gel gradients by the method of Laemmli [30]. Samples were diluted with an equal volume of sample buffer and then heated at 95 °C for 5 min. The gels were fixed and stained with Coomasie Brillant Blue R-250, followed by destaining in

45% methanol and 7% acetic acid. Each sample lane was cut into 2 mm slices. The slices were digested in 900 μl of 30% H_2O_2 at 50 °C in capped vials overnight. Twenty milliliters of scintillation fluid (Ready-Safe, Beckman Coulter Inc.) were then added to each vial prior to determination of total radioactivity by a Packard scintillation counter. The yield of photolabeling was calculated by adding the total radioactivity in the [^{3}H]DEX-labeled peak (minus baseline background) and expressing it as a percentage of total [^{3}H]DEX-binding complexes present in the initial non-photolabeled sample, as previously determined. Gels were calibrated with the following [^{14}C]-labeled proteins: myosin (220 kDa); phosphorylase b (97 kDa); BSA (66 kDa); ovalbumin (45 kDa); carbonic anhydrase (30 kDa); trypsin inhibitor (20.1 kDa) and, lysozyme (14.3 kDa). The molecular masses of radioactive macromolecules were estimated by their mobilities relative to standard proteins of known molecular masses.

2.12. Protein measurement

Proteins were measured by the Bio-Rad D_c (detergent compatible) method (Bio-Rad Laboratories, CA), using BSA as standard.

2.13. Data analysis

Mathematical analysis of the data was by the KINETIC, EBDA and LIGAND-PC curve fitting programs [31]. Statistical comparisons for each of the binding and kinetic parameters versus the control sample were made by paired Student's *t*-test. Significance of differences among groups was tested by the Student–Newman–Keuls test. The data are expressed as the mean ± S.E. Statistical significance was reported if $P < 0.05$ was achieved.

3. Results

3.1. The endoplasmic reticulum-associated glucocorticoid-binding protein is a multimeric protein with a molecular mass of at least 135 kDa

We have already shown that the [^{3}H]DEX-binding activity of LAGS is associated with an integral membrane protein bound to rat liver endoplasmic reticulum and an estimated molecular mass of at least 134 kDa [14]. To further characterize its hydrodynamic properties, we have now solubilized LAGS in a functional form using the non-ionic detergent TX100. The modification of the phospholipid environment of the LAGS does not affect protein-binding affinity, as it is suggested by the estimated K_d values in TX100 and crude membranes (85 nM versus 100 nM). The Scatchard plot of the [^{3}H]DEX-binding to solubilized proteins revealed similar B_{max} level in TX100 extracts and in membranes (11 pmol/mg protein versus 12 pmol/mg protein). Analysis of the Scatchard plots of CHAPS-extracts indicates that solubilized-LAGS is composed of a unique class of binding sites, just as for membrane-bound LAGS [13] and CHAPS-solubilized LAGS [14]. This hypothetical model is reinforced by the fact that both association and dissociation kinetics fit with a monophasic interaction of [^{3}H]DEX with LAGS (data not shown).

To determine their molecular size, TX100 extracts were first chromatographed on a Sephacryl S-300 column. We used Acker's method [26] to construct the calibration graphs, obtaining a good linear relationship between Stokes radius and partition coefficient (Fig. 1, inset). Stokes radii of complexes were determined by interpolation in the calibration plots. As was previously shown with the CHAPS-solubilized complex [14], the [^{3}H]DEX-binding activity was found in one well-defined peak of TX100-solubilized proteins. When 60 μM unlabeled DEX was added with [^{3}H]DEX,

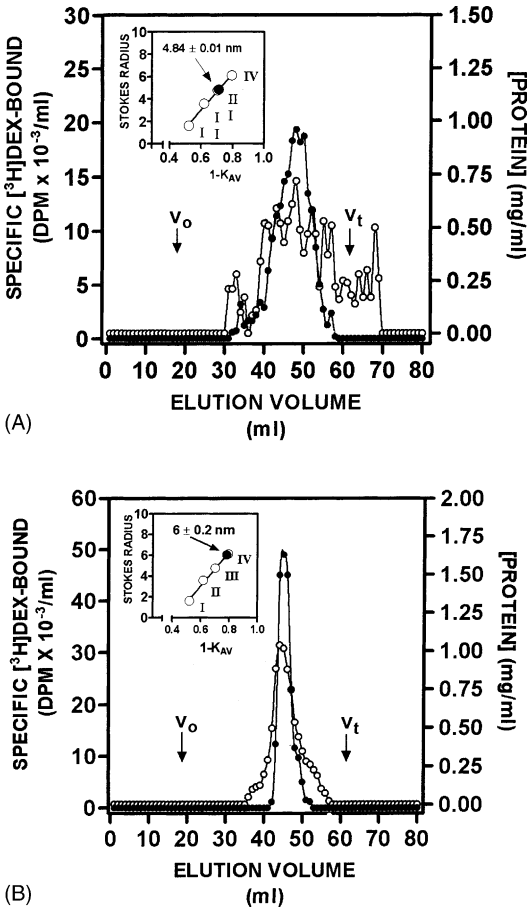

Fig. 1. Sephacryl S-300 chromatography of detergent-solubilized LAGS from rat liver. Aliquots (6–7 mg protein/ml) of CHAPS (A) or Triton X-100 (B) solubilized LAGS were loaded on a Sephacryl S-300 column. The [^{3}H]DEX-binding activity (●) and protein content (○) were then measured in each fraction (1 ml), as described under Section 2. The column was calibrated with proteins of known Stokes radius (inset), and the following protein markers were used: (I) carbonic anhydrase; (II) BSA; (III) alcohol dehydrogenase; and (IV) apoferritin. Results are representative of five separate experiments with similar results. V_0 and V_t indicate the void volume and total volume of the column, respectively.

Table 1
Hydrodynamic parameters of solubilized [^{3}H]DEX-binding protein from adult male rat liver microsomes

Parameter	CHAPS	Triton X-100
Stokes radius (nm)	4.84 ± 0.02 (3)[a]	6 ± 0.3 (3)
$s_{20,w}$		
In H_2O	6.50 ± 0.03 (3)	3.65 ± 0.25 (3)
In D_2O	6.26 ± 0.42 (2)	3.45 ± 0.15 (2)
s (S)[b]	6.383	3.55
Partial specific volume (ml/g)[c]	0.74	0.74
Molecular mass (kDa)[d]	134.2 (133.9–134.5)[e]	92.7 (89.6–95.8)[e]

[a] Data represent mean ∀ S.D. of the number of experiments indicated between parentheses.
[b] Data were calculated from all possible pareated combinations between data obtained from gradients in H_2O and D_2O.
[c] It is assumed that partial specific volume of LAGS complexes is identical to that of standard proteins.
[d] See Section 2 for the calculations.
[e] Values of this range were calculated using 1 ± S.E.M.

the peak of [^{3}H]DEX-binding activity was completely abolished, supporting the specificity of the labeling. However, the Stokes radius of the [^{3}H]DEX-binding activity was higher in the TX100-solubilized complex than that of the CHAPS-solubilized complex (Table 1). The hydrodynamic properties of LAGS were further investigated by subjecting the detergent solubilized samples to ultracentrifugation on sucrose density gradients. The profiles obtained after sucrose gradients made up in H_2O are shown in Fig. 2. A single peak of [^{3}H]DEX-binding activity was resolved in 4–20% linear sucrose gradient containing 4.5 mM TX100. The $s_{20,w}$ of the detergent-LAGS complexes determined in H_2O were nearly the same as those calculated from gradients made up with D_2O (Table 1), thus suggesting that these complexes and the calibrating proteins have a very similar partial specific volume. A typical feature of the protein–TX100 complexes is a combination of a low sedimentation quotient and high Stokes radius, as described here. This is mainly caused by the high partial specific volume of the complex due to the bound TX100 (partial specific volume of TX100 = 0.908), but asymmetry and abnormal hydration of the complex may also contribute [30]. Finally, the molecular mass of LAGS–detergent complexes was then calculated from gel filtration and ultracentrifugation experiments, as described under Section 2. Taken together, these findings show that, in the presence of the detergent TX100, LAGS has a molecular mass of at least 93 kDa, which is smaller than the previously reported for the CHAPS-solubilized LAGS (i.e., 135 kDa).

3.2. Photoaffinity labeling identification of thyroid hormone-regulated peptides in endoplasmic reticulum-associated glucocorticoid-binding protein

We have previously described that membranes from male rat liver endoplasmic reticulum contain both ST-binding

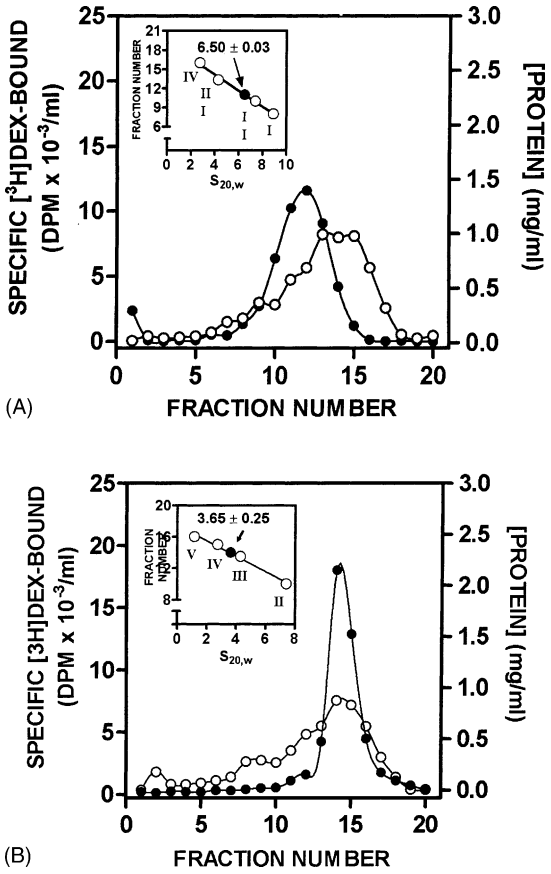

Fig. 2. Hydrodynamic characterization of the detergent-solubilized LAGS from rat liver. Aliquots (7–8 mg protein/ml) of CHAPS (A) or Triton X-100 (B) solubilized LAGS were loaded onto 21–36% or 4–20% sucrose gradients for both detergents, respectively, and centrifuged at 350,000 × g for 16 h. Fractions (200 μl) were collected and analyzed for [^{3}H]DEX-binding activity (●) and total protein concentration (○), as described under Section 2. The calibration plots were constructed by ultracentrifuging proteins of known $s_{20,w}$ under the same conditions (insets). The $s_{20,w}$ value LAGS was estimated from this standard curve. The following protein markers were used: (I) β-amylase; (II) alcohol dehydrogenase; (III) BSA; (IV) carbonic anhydrase; and (V) cytochrome *c*. Results are representative of five separate experiments with similar results.

peptides (i.e., STBP) [10] and glucocorticoid-binding peptides (i.e., LAGS) [14]. Interestingly, STBP is functionally related with LAGS, and these steroid-binding proteins are under regulation by pituitary hormones. However, unlike LAGS, STBP is present from birth, suggesting that ST-binding activity and DEX-binding activity are under different hormonal regulation in vivo. Thus, we considered it of interest to study whether hypothyroidism, an experimental model in which at least two hormones (i.e., GH and thyroid hormone) involved in LAGS regulation are decreased, also affected the STBP.

In accordance with previous reports [17], liver microsomes from TX adult male rats had 10% of [^{3}H]DEX-binding activity compared to that from intact animals (1.3 pmol/mg protein versus 13 pmol/mg protein) (Fig. 3). The administration of T3 (2.5 μg/100 g BW day) to TX rats caused

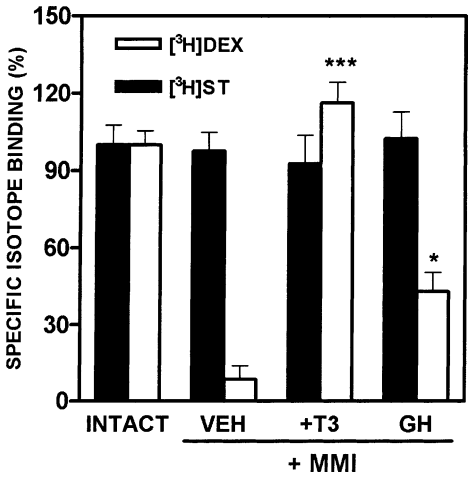

Fig. 3. The membrane-associated glucocorticoid-binding activity (LAGS) and the membrane-associated ST biding activity (STBP) are differently regulated by thyroid hormones. Hypothyroidism was induced in adult Sprague-Dawley rats by MMI treatment. After 21 days of MMI treatment, the animals were injected GH or T3. The doses were 100 μg hGH/rat day, divided in three daily injections for 7 days, 2.5 μg T3/100 g BW day for 5 days. The animals were killed 24 after the last injection, and their livers were quickly removed and processed as described in Section 2. Control rats received only the appropriate vehicle (VEH). The values obtained in intact rats are shown as a reference. Each point is the mean ± S.E.M. of at least five separate experiments. (✱) $P < 0.05$ vs. vehicle alone; (***) $P < 0.001$ vs. vehicle alone.

full recovery of the [^{3}H]DEX-binding activity. However, GH administration (100 μg/rat day) induced only 50% intact rat levels. Surprisingly, TX did not modify the [^{3}H]ST-binding activity, suggesting that neither thyroid hormone nor probably GH play a role in endocrine regulation of the STBP. Thus, to characterize the effects of T3 and GH on membrane-associated glucocorticoid-binding peptides, microsomes from TX adult male rats treated with GH or T3 at the doses indicated above were CHAPS-solubilized and irradiated in the presence of 300 nM [^{3}H]DEX. As expected, SDS-PAGE of covalent labeled LAGS from adult liver microsomes gave two peptides of 53 and 37 kDa, respectively ([14] and Fig. 4A). However, these peptides were dramatically reduced in microsomes from untreated TX rats (Fig. 4B). Interestingly, both GH (Fig. 4C) and T3 (Fig. 4D) increased the level of 53 kDa [^{3}H]DEX-binding peptide more efficiently than the level of the 37 kDa peptide.

Thus, photoaffinity labeling of glucocorticoid-binding peptides of CHAPS-solubilized microsomes with [^{3}H]DEX has allowed the identification of two peptides of 53 ± 4 and 37 ± 2 kDa ([14] and Fig. 4A). The presence of two glucocorticoid-binding peptides and the fact that they are regulated by hormones prove that the LAGS is an heterooligomeric protein with constituent peptides under regulation by physiological stimuli in vivo. Furthermore, our results demonstrate that ST-binding activity and DEX-binding activity are associated with different steroid-binding peptides. These data also may well support the allosteric interaction of ST and its metabolites (see below) with this membrane-associated glucocorticoid-binding protein.

3.3. Unlike cytosolic GR, the endoplasmic reticulum-associated glucocorticoid-binding protein is a high-affinity receptor for 16β-hydroxylated stanozolol

There is evidence to support a role for antiglucocorticoid mechanism in the anabolic effects of pharmacological 17α-AA regimens [1–4]. Thus, we hypothesize if the pharmacological effects of ST on liver could be mediated by the modulation of glucocorticoid interaction with cytosolic GR, LAGS or both proteins. As expected, 17α-methyltestosterone binds to the GR [33]. However, neither ST nor its major hydroxylated metabolites (i.e., 16β-OHST and 3-OHST) nor the structural analog danazol (DA) bind to GR (Fig. 5A). In contrast, 16β-hydroxylation of ST shifts its affinity for LAGS from 30 to 13 nM. In contrast, 3′-hydroxylation of ST suppresses the capability of LAGS to bind ST (Fig. 5B).

To further explore the mechanism of interaction of 16β-OHST with LAGS, we performed dissociation experiments of CHAPS-solubilized [^{3}H]DEX-LAGS complex in the presence or absence of this 17α-AA. Once [^{3}H]DEX-binding to solubilized LAGS reached equilibrium, the dissociation kinetics of [^{3}H]DEX from LAGS was carried out by adding 200-fold excess of unlabeled DEX. The incubation was continued at 4 °C, and the reaction was stopped by the addition of DCC to the incubation mixture at the times specified. In keeping with previous work [14], dissociation experiments showed that specific [^{3}H]DEX-binding to solubilized LAGS was reversible, and a monoexponential model most closely explained the dissociation (Fig. 6 and Table 2). Under such conditions, 16β-OHST, ST or DA significantly increased the dissociation rate constant of the CHAPS-solubilized [^{3}H]DEX-LAGS complex. Thus, the ability of these steroids to accelerate the dissociation of the [^{3}H]DEX-LAGS complex strongly suggests that these 17α-AA, through their binding to a second site, cause an inhibition of the glucocorticoid-binding activity of the LAGS through a negative allosteric mechanism. Furthermore, our findings show that the allosteric site remains associated with LAGS even when this protein is removed

Table 2
Effects of 17α-alkylated androgens on dissociation rate of [^{3}H]DEX from LAGS

Inhibitor	K_{-1} ($min^{-1} \times 10^{-4}$)
DEX	60 ± 5
DA	84 ± 4
ST	127 ± 7
16β-OHST	166 ± 10

The kinetic program was used in the analysis of the data. A monoexponencial fit was selected for DEX, DA, ST, and 16β-OHST. Each value is the mean ± S.E. of six separate experiments. K_{-1}, dissociation rate constant.

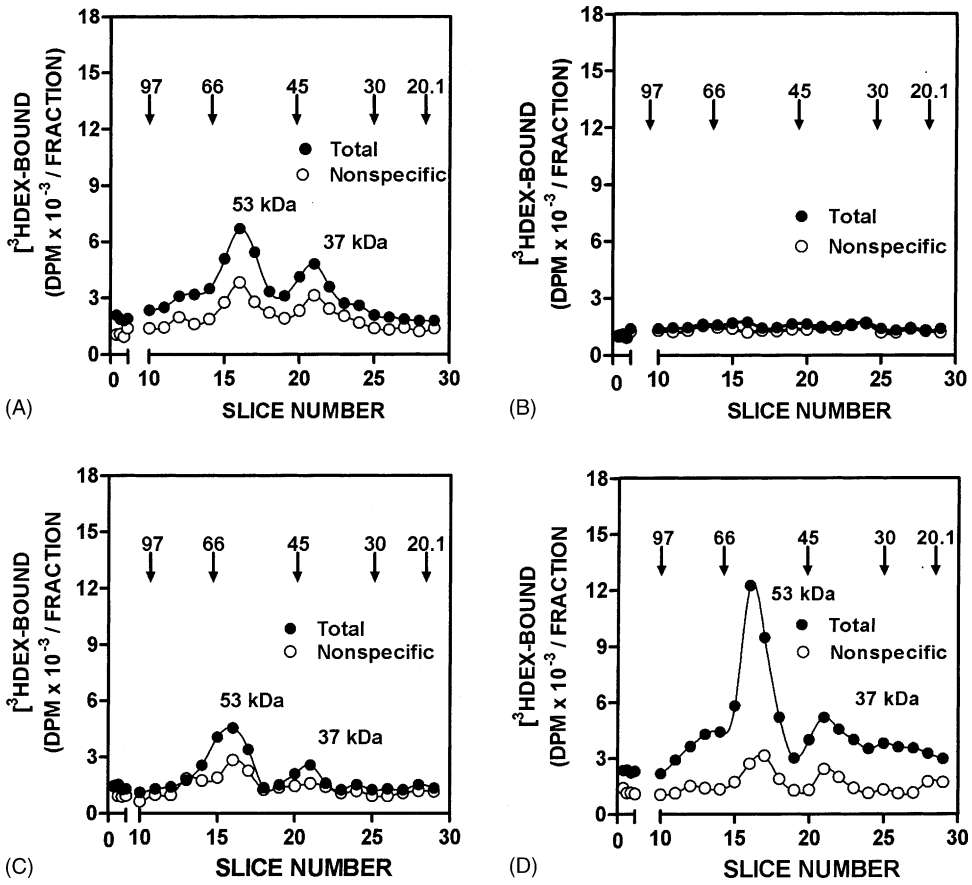

Fig. 4. SDS-PAGE of thyroid hormone-dependent expression of membrane-associated glucocorticoid-binding peptides from rat liver microsomes. CHAPS-solubilized microsomal proteins from liver of 2–3-month-old (A), TX (B), GH (C), and T3 (D) treated male rats were photoaffinity labeled with [^{3}H]DEX (300 nM), as described under Section 2. The covalent [^{3}H]DEX-binding proteins were concentrated by centrifugation with Centriplus YM, and dissolved in sample loading buffer. Aliquots (3 mg of protein) were electrophoretically separated on a 10–20% polyacrylamide gel gradient under reducing conditions. The positions of [^{14}C]-labeled protein markers are shown by arrows. Molecular masses for the markers are given in kDa. The data are representative of four independent experiments performed with similar results.

from the membrane environment. Since hydroxylation of ST is an important enzymatic reaction in the liver, our data also suggest that 16β-hydroxylation of ST might contribute to the formation of active steroid metabolites, just as it has been demonstrated for other steroids [34].

4. Discussion

Although the liver is an important target for 17α-AA, its mechanism of action is a subject of some controversy. To further characterize the mechanisms whereby 17α-AA exerts its effects on the liver we have characterized the hydrodynamic properties of the endoplasmic reticulum-associated glucocorticoid-binding protein LAGS. Photoaffinity labeling of LAGS has been used to identify the constituent thyroid hormone-regulated peptides. The results presented constitute the first experimental evidence that LAGS, unlike cytosolic GR, is a high-affinity receptor for the major urinary metabolite of ST, 16β-OHST. Like its precursor, 16β-OHST is a negative allosteric inhibitor of LAGS, suggesting that 16β-hydroxylation of ST might contribute to modulating glucocorticoid activity in the liver.

First, size exclusion chromatography and sucrose density gradient ultracentrifugation of TX100-solubilized LAGS showed the existence of a single [^{3}H]DEX-binding component associated with endoplasmic reticulum membranes. Since no significant difference was observed between the sedimentation coefficient values measured in H_2O and D_2O, it has been assumed that the protein binds the same amount of detergent in each medium and that the partial specific volume of LAGS is similar to that of globular proteins used as markers [28]. Therefore, in making the calculations, a partial specific volume of 0.74 cm^3/g was used, as this volume was selected by Martin and Ames as being representative for most proteins in their sucrose density centrifugation studies [32]. On the basis of the cumulative values obtained from hydrodynamic studies, the molecular mass of the TX100-solubilized LAGS was estimated to be at least 90 kDa. Surprisingly, this predicted molecular mass is significantly smaller than that reported for CHAPS-solubilized LAGS (i.e., 135 kDa) [14]. This

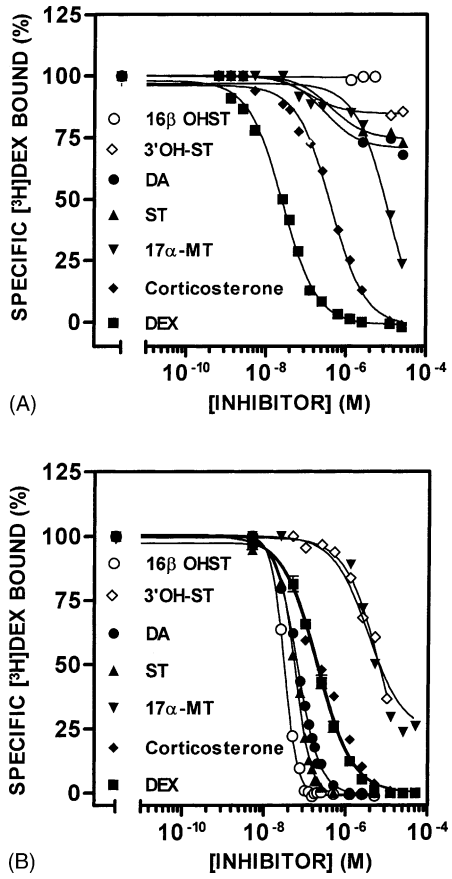

Fig. 5. Stanozolol and ST metabolites do not bind to cytosolic GR, but are high-affinity ligands for the membrane-associated glucocorticoid-binding protein LAGS. Cytosol (A) or CHAPS-solubilized microsomal suspensions (B) were incubated with [^{3}H]DEX (50 nM), in the absence (vehicle) or in the presence of 12 increasing concentrations of unlabeled inhibitor (from 10 nM to 50 μM). Incubations were continued overnight at 0–4 °C. The samples were then treated with DCC and counted as described under Section 2. Each curve represents the mean of the duplicate from a single experiment representative of at least five separate experiments. 17α-MT, 17α-methyltestosterone.

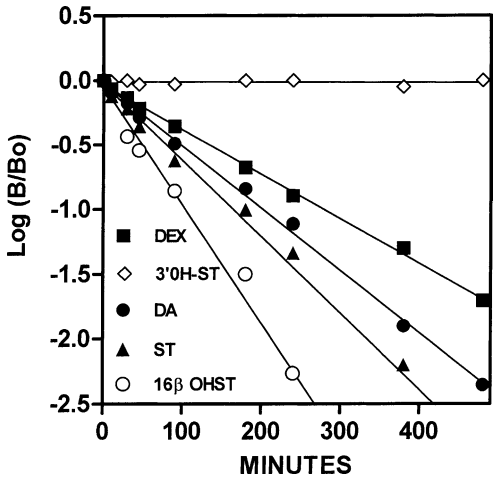

Fig. 6. Stanozolol and ST metabolites are negative allosteric modulators of glucocorticoid-binding to the membrane-associated glucocorticoid-binding protein LAGS. The CHAPS-solubilized microsomal samples were incubated with [^{3}H]DEX (150 nM) for 12 h. Nonspecific binding was determined in parallel incubations with a 200-fold excess of unlabeled DEX. The dissociation phase was initiated with 30 μM of the indicated unlabeled steroids. At the indicated times, duplicate samples were treated with DCC and counted, as described in Section 2. The data are representative of four separate experiments. B_0, specific [^{3}H]DEX bound at time zero; B, specific [^{3}H]DEX bound at time t.

discrepancy may be tentatively ascribed to the absence of TX100 insoluble material in our hydrodynamic studies, which in turn could explain why the TX100-extracted complexes are smaller in sedimentation value (i.e., 3.65 S versus 6.50 S) and the predicted molecular mass of LAGS (i.e. 90 kDa versus 135 kDa). Interestingly, one of the biochemical definitions of caveolae is that they resist TX100 solubilization [35], and caveolae appear to be present in endoplasmic reticulum fractions as well as in plasma membranes. Furthermore, caveolae are the location of many important signalling molecules in diverse cell types, and it is suggested that the interactions of important signaling partners occur in this compartment [36]. Whether DEX-binding to rat liver endoplasmic reticulum is caveolae-linked is still under research in our lab.

A membrane form of GR (mGR) has been described in specific cell types [37]. Although neither has been identified as mGR, liver plasma membranes express two glucocorticoid-binding proteins. The first is a cortisol-binding protein composed at least in part of two polypeptides of 52 and 57 kDa [38], and the second an active transmembrane transporter of corticosterone that does not belong to the ATP-binding cassette/multidrug resistance transporter superfamily [39]. However, the different subcellular localization, together with the fact that plasma membrane-glucocorticoid-binding proteins exhibit different binding characteristics when compared to LAGS, an endoplasmic reticulum-associated glucocorticoid-binding protein, make it unlikely that these glucocorticoid-binding proteins and LAGS are the same entity. Furthermore, by affinity labeling with DEX mesylate followed by SDS-PAGE, the existence of two DEX mesylate-binding peptides of 45 kDa has been characterized in liver membranes [40]. In contrast, affinity labeling with DEX mesylate (unpublished results) or photoaffinity labelling with DEX followed by SDS-PAGE of CHAPS-solubilized LAGS ([14], and this paper) prove the existence of a DEX-binding protein composed at least in part of two polypeptides of 53 and 37 kDa. It is possible that the 37 kDa peptide may result from the proteolytic cleavage of the 53 kDa peptide, despite the cocktail of proteolysis inhibitors used; the fact that the molecular mass estimated (i.e., 90 kDa) for TX100-solubilized LAGS by hydrodynamic studies is quite close to that obtained by SDS-PAGE of photoaffinity-labeled LAGS makes proteolysis less likely. Furthermore, the endocrine regulation studies provide evidence of a variable amount of each peptide under different treatment conditions. These data, together with those previously reported by our lab [14], definitely prove that [^{3}H]DEX-binding peptides are associated with a

heterooligomeric protein with a molecular mass of at least 90–135 kDa.

In rat liver endoplasmic reticulum, a membrane-progesterone-binding protein (mPBP) has also been described [41]. This is a 200 kDa oligomeric protein composed at least in part of 28 and 56 kDa polypeptides. It has been shown that the antipsychotic drug haloperidol inhibits the binding of progesterone to mPBP, and progesterone inhibits the binding of haloperidol to the sigma-like haloperidol receptor, thus giving rise to the possibility that the mPBP is, at least in part, the sigma-like haloperidol receptor. Interestingly, we have described that LAGS is also a high molecular mass protein composed at least in part of 37 and 53 kDa polypeptides. However, DEX does not bind to mPBP, nor haloperidol to LAGS (unpublished results), which makes it very unlikely that LAGS and mPBP are the same entity.

The endoplasmic reticulum-associated STBP is functionally related to LAGS [10]. However, several lines of evidence suggest that endoplasmic reticulum-associated ST-binding peptides differ from glucocorticoid-binding peptides. First, photoaffinity labeling with [^{3}H]ST followed by SDS-PAGE shows three peptides of 55, 31, and 22 kDa. When these peptides were resolved under non-reducing SDS-PAGE, we detected a single 104 kDa peptide, thus suggesting the existence of disulfide bonds present within the peptides [10]. In contrast, we obtained identical SDS-PAGE profiles of covalently [^{3}H]DEX-labeled peptides when they were separated both under reducing and non-reducing SDS-PAGE [unpublished results]. Second, [^{3}H]ST interaction with the STBP suggests a complex mechanism of interaction that contrasts with that for the interaction of [^{3}H]DEX with LAGS ([13], and this paper). Moreover, the STBP shows an extremely narrow pharmacological profile, being selective for ST, DA, and 16β-OHST (data not shown), among more than 100 steroidal and non-steroidal compounds assayed, including all those capable of displacing DEX binding to LAGS [42]. Third, the STBP has a longer half life than that of LAGS (data not shown). Fourth, the STBP ligand ST is an allosteric modulator of the DEX-LAGS complex. Fifth, unlike LAGS, STBP is already present at birth [10]. Finally, in the present work we have proven that neither T3 nor GH regulate STBP; whereas these hormones are essential regulators of LAGS.

The nature and biological roles of membrane-glucocorticoid-binding proteins are still a matter of intense debate. Given that DEX-binding activity is insensitive to cytochrome P450 inhibitors or sulfated steroids [13], as well as its apparent molecular mass (90–135 kDa), it is unlikely that the LAGS is a steroid-metabolizing enzyme, such as the cytochrome P450 family [43] (48–56 kDa) or flavin monoxygenases (55–59 kDa) [44]. In addition, members of a family of GTP-binding proteins recently identified in the endoplasmic reticulum membrane possess similar molecular masses (20–30 kDa) to the 37 kDa DEX-binding peptide [45]. Some of them are also associated in larger structures (about 100 kDa) [46]. Whether DEX-binding to liver membranes is sensitive to GTP and GTP analogues is still under research in our lab.

The specific contribution of antiglucocorticoid mechanisms to the cellular effects of pharmacologic androgen regimens is still controversial. In vitro studies suggest that testosterone has a very high binding affinity for the GR [47], and that testosterone exerts anabolic activity by acting as an antagonist to endogenous glucocorticoids [48]. 17α-AA have been reported to bind, albeit at low affinity, to GR ([4], and this paper), which might be responsible in part for antiglucocorticoid effects of these steroids on tissues. Interestingly, ST exerts an anticatabolic action in male rats by the partial inhibition of the catabolic response to a high dose of corticosterone [49]. Surprisingly, our results demonstrate that neither ST nor ST metabolites bind to GR. However, they are both high-affinity ligands and allosteric inhibitors of LAGS. These findings make it unlikely that ST modulates glucocorticoid activity in liver through direct interaction with the cytosolic GR. Thus, in contrast to antiglucocorticoid hypothesis, the interaction of ST (or 16β-OHST) with LAGS would effectively increase glucocorticoid availability to the cytosolic GR. Interestingly, LAGS can be saturated under physiological, pathological, and pharmacological conditions [50] and this protein is associated with higher levels of DEX being required to induce tyrosine aminotransferase in vivo [15]. In addition, although the importance of 16β-hydroxylation in a physiological or pharmacological context remains to be established, our data seem to suggest that this enzymatic reaction could contribute to modulating ST activity in the liver.

Acknowledgements

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