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Solubilization and photoaffinity labeling identification of glucocorticoid binding peptides in endoplasmic reticulum from rat liver☆

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Abstract

Steroid-binding proteins unrelated to the classical nuclear receptors have been proposed to play a role in non-genomic effects of steroid hormones. We have previously described that the low-affinity glucocorticoid binding protein (LAGS), present in the endoplasmic reticulum of the male rat liver, has pharmacological and biochemical properties different from those of nuclear receptors. The LAGS is under multihormonal regulation and binds glucocorticoids, progestins, and synthetic steroids but is unable to bind either estradiol, testosterone, or triamcinolone acetonide. In this study, we have solubilized the LAGS and investigated their pharmacological and hydrodynamic properties and their peptide composition. We found that LAGS is an integral protein bound to the endoplasmic reticulum. CHAPS provided its optimal solubilization without changes in its pharmacological properties. Hydrodynamic properties of LAGS showed that it has a molecular mass of at least 135 kDa. SDS-PAGE of covalently-labeled LAGS showed that [^{3}H]dexamethasone binds two peptides of 53 and 37 kDa, respectively. Thus, the LAGS appears as an oligomeric protein under multihormonal regulation. The availability of solubilized LAGS and the fact that it can be induced in vivo represent major steps toward purification and understanding the functional significance of this unique steroid-binding protein.

Keywords: Membranal steroid receptors; LAGS; Glucocorticoids; Liver; Photoaffinity

1. Introduction

Recognition of the complexity of steroid signaling has substantially increased over the last years. In their genomic role [1], steroids enter the cell and bind members of the superfamily of ligand-regulated transcription factors. However, steroids also exert non-genomic effects that occur independently of gene transcription [2,3]. Recent studies suggest that steroid hormones use receptors on cellular membranes both to gain access to the intracellular compartment [3] and to modulate cellular functions. Typically, these effects are initiated at the plasma membrane and result in the regulation of membrane protein-mediated intracellular signaling pathways [3]. On the other hand, one of the major controversies about the non-genomic effects of steroids has been the nature of the membrane steroid-binding protein(s). Several mechanisms could account for the non-genomic effects of steroid hormones [2,3]. These include: (1) non-specific steroid effects on the lipid bilayer; (2) classical nuclear receptors located at the membrane; and (3) steroid-binding proteins unrelated to the classical nuclear

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Abbreviations: 17α-AA, 17α-alkylated androgen derivatives; AR, androgen receptor; DA, danazol; DCC, dextran T70-coated charcoal; DTT, dithiothreitol; DEX, dexamethasone; LAGS, low-affinity glucocorticoid binding sites; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; ST, stanozolol; STBP, [^{3}H]stanozolol-binding protein

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receptors located at the plasma membrane or intracellularly.

We have recently demonstrated that the liver endoplasmic reticulum contains a highly specific binding protein for the 17α-alkylated androgen derivatives (17α-AA) stanozolol (ST) and danazol referred to as stanozolol-binding protein (STBP) [4]. This is an oligomeric protein whose peptides are under hormonal regulation by pituitary hormones and ethinylestradiol [4], it is present in human tissues (unpublished data), and it can be saturated with the doses of these 17α-AA steroids used for treatment of various diseases and often abused by athletes [5,6]. 17α-AA exert important effects on the liver, and many of the pathologic conditions associated with their prolonged use emanate from the adverse effects of these steroids on hepatic gene expression [5]. Their genomic effects are generally thought to act through their binding to the nuclear androgen receptor (AR) acting as androgen agonists [5]. It is believed that they may also act as antiglucocorticoids by competing with endogenous ligands for their binding to the glucocorticoid receptor (GR) [5]. However, 17α-AA also exert non-genomic effects that occur independently of gene transcription [2,3].

We have suggested that some of the effects of STBP's ligands on liver may appear through their allosteric modulation of the glucocorticoid-binding activity of the low-affinity glucocorticoid binding protein (LAGS) [4,7,8]. The LAGS is an endoplasmic reticulum-associated glucocorticoid binding protein present in male rat liver capable of interacting with glucocorticoids and 17α-AA with a lower affinity than that exhibited by the respective receptors for these hormones [7–9]. Interestingly, the LAGS is unable to bind either natural estrogens or natural androgens. LAGS is under multihormonal regulation by glucocorticoids [10], thyroid hormones [10], growth hormone [11], 17α-alkylated estrogens [9], 17α-AA [7,9], and Vitamin A [12]. Thus far, the evidence suggests that most of the steroids that this protein is capable of binding have powerful effects on the liver and that 17α-AA can interact and modulate the LAGS' activity under physiological or pharmacological conditions.

Thus, to further characterize the mechanisms of 17α-AA effects on the LAGS-associated glucocorticoid-binding activity, we describe in the present work the optimal conditions to solubilize a functional LAGS from male rat liver endoplasmic reticulum. Thereby, we have been able to analyze its hydrodynamic properties by molecular sieving and sucrose density gradient ultracentrifugation. Photoaffinity labeling, a powerful tool for the identification and characterization of steroid-specific mediators of biological and pharmacological phenomena [13], has been used to identify the glucocorticoid binding peptides of this oligomeric protein. The availability of solubilized LAGS and the fact that it can be induced in vivo [9–12] represent major steps toward purification and establishing the functional significance of this unique steroid-binding protein.

2. Materials and methods

2.1. Reagents

[^{3}H]Dexamethasone ([^{3}H]DEX; 36–45 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Ready safe scintillation cocktail was purchased from Beckman Instruments Inc. (Palo Alto, CA). Unless otherwise indicated, the products cited in this work were purchased from Sigma.

2.2. Animals

Adult (2–3-month-old) male Sprague–Dawley rats were used throughout these experiments. All the animals were sacrificed by decapitation, and their livers were removed quickly 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 microsomes

All the 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 (mM): Tris–HCl, 50; sodium molybdate, 10; magnesium chloride, 5; dithiothreitol (DTT), 2; sucrose, 0.25; phenylmethylsulfonyl fluoride, 1; EDTA, 1; and 1 μg/ml soybean trypsin inhibitor, pH 7.5. Homogenates were centrifuged at 17,000 × g for 15 min to separate nuclear and mitochondrial fractions. The supernatant was centrifuged at 105,000 × g for 1 h, and the pellets were resuspended in TMMDSI buffer and recentrifuged again at 105,000 × g. The centrifuged pellet was resuspended in an appropriate volume of TMMDSI buffer to give a protein concentration of 2–3 mg/ml. The microsomal suspensions were used for the binding assays.

2.4. Radioligand binding assays

For LAGS' activity, 200 μg of microsomal suspensions or solubilized proteins were incubated overnight at 0–4 °C, in duplicate, with increasing concentrations (from 10 to 500 nM final concentration) of [^{3}H]DEX. Non-specific binding for LAGS activity was measured in 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 non-specific binding represented less than 10% of the total binding; higher concentrations of DCC did not achieve greater effectiveness. Non-specific binding showed an excellent linearity in the overall 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. The DCC was eliminated by centrifugation.

2.5. Subcellular fractionation of rat liver membranes

For examining the subcellular distribution of the [^{3}H]DEX-binding activity, livers obtained from two adult male rats were perfused with ice-cold physiological saline, as previously described, and were homogenized in four volumes of ice-cold TMMDSI buffer. All subsequent steps were performed at 4 °C. The homogenate was filtered through a nylon blotting cloth (pore size 50 μm), and the filtrate was centrifuged twice at 1000 × *g* for 10 min. The nuclear- and plasma membrane-enriched fractions were obtained from this pellet [14], according to the procedure using a Percoll gradient [15]. The 1000 × *g* supernatant was centrifuged at 10,000 × *g* for 15 min. Half of this pellet, approximately, was suspended in TMMDSI buffer (total volume 6 ml), and 1.5 ml of suspension were layered on 10 ml of isoosmotic 40% Percoll solution ($\rho \sim 1.08\,g/cm^3$). This was then centrifuged at 60,000 × *g* (70.1Ti fixed-angle rotor, Beckman Instruments, Fullerton, CA) for 30 min. Thus, three fractions—an upper portion (peroxisome fraction, $\rho < 1.070\,g/cm^3$); a lower one (mitochondria-enriched fraction, $1.070 < \rho < 1.128\,g/cm^3$); and a portion adjacent to the bottom (lysosome-enriched fraction, $\rho \sim 1.123\,g/cm^3$)—were roughly separated. Each fraction was washed three times with TMMDSI by centrifugation at 16,000 × *g* for 20 min. The 10,000 × *g* supernatant, previously obtained, was centrifuged at 105,000 × *g* for 60 min, and the resultant supernatant was recentrifuged under the same conditions and was 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 the modified Rostchild method [16]. Briefly, the pellet was resuspended in TMMDSI containing sucrose sufficient to give 0.15 M, final concentration; 7.5 ml of this solution was layered over a 2 ml cushion of TMMDSI–1.3 M sucrose and centrifuged 100 min at 320,000 × *g*. After this, the RER fraction was recovered as a sediment in the bottom of the tube, whereas the SER formed a band at the interface that, once diluted 1:3 with ice-cold TMMDSI, was recovered by means of centrifugation at 105,000 × *g* for 60 min. All fractions above obtained 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) [17].

2.6. Solubilization of LAGS

Unless otherwise indicated, extraction of proteins was accomplished by incubating microsomes (3 mg/ml) in the presence of the zwitterionic detergent CHAPS (7.5 mM) in TMMDSI–0.1 M KCl buffer for 2 h, by gentle agitation at 4 °C. Insoluble materials were 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 1 M KCl–TMMDSI for 30 min at 0 °C, and the extracted microsomes were collected by ultracentrifugation and solubilized with detergents, as described above.

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 [18]. Briefly, for the separation of proteins a cushion of 6% (w/v) sucrose 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 loaded on this sucrose cushion, and the tube was incubated for 3 min at 30 °C for condensation. 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 received 0.3% fresh Triton X-114. After dissolution of the surfactant at 0 °C, the mixture was again loaded on the sucrose cushion used previously, incubated for 5 min at 30 °C for condensation, and centrifuged on the previous detergent phase. At the end of the separation, the aqueous phase was rinsed with 1% Triton X-114 in a separate tube without a sucrose cushion. The detergent phase of this last condensation was 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

Approximately 12–14 mg of 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 containing 10 mM CHAPS. 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: BSA (3.55 nm); alcohol dehydrogenase (4.75 nm); and catalase (5.21 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 coefficient (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 [19].

2.9. Sucrose density gradient ultracentrifugation

The sedimentation coefficient ($s_{20,w}$) and the partial specific volume of LAGS–detergent complexes were determined by centrifugation through sucrose density gradients in H_2O or D_2O [20]. Linear gradients (4 ml) of 21–36% (w/v) sucrose were prepared in H_2O and 4–20% in D_2O, containing TMMDSI buffer, 7.5 mM CHAPS and 1 M KCl. The gradients were allowed to cool at 4 °C, and 150 μl (about 1.6 mg of protein) of each sample were layered on top of the gradients. On parallel gradients the following standard proteins were sedimented in the same run: carbonic anhydrase ($s_{20,w} = 2.75$); BSA ($s_{20,w} = 4.3$); 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 [21], 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 LAGS–detergent complexes were calculated from their Stokes radius, partial specific volume, and $s_{20,w}$ value using Svedberg's equation [21]. Data are given as mean ± S.D.

2.10. Photoaffinity labeling of LAGS

[^{3}H]DEX (400 nM) was incubated in the dark with 200 μg of 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 six-well plates (15 mm i.d.) and irradiated for 20 min at 0 °C with a 254 UV lamp (Stratagene) at a distance of 100 mm above plate. Non-specific binding and photolabeling were defined with 60 μM DEX. Photolabeled samples were concentrated by centrifugation with Amicon YM-100. 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. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of photolabeled LAGS

SDS-PAGE was carried out on polyacrylamide gel gradients by the method of Laemmli [22]. 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 Brilliant Blue R-250, followed by destaining in 45% methanol and 7% acetic acid. Each sample lane was cut into 3 mm slices. The slices were digested in 1 ml of 30% H_2O_2 at 50 °C in capped vials for 48 h. Twenty milliliters of scintillation fluid (NCS, Amersham) were then added to each vial prior to determination of total radioactivity by a Packard scintillation counter. The yield of photolabeling was calculated by summing 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 using the following [^{14}C]-labeled proteins: BSA (66.2 kDa); ovalbumin (45 kDa); glyceraldehyde 3-P (36 kDa); carbonic anhydrase (29 kDa); β-casein (23.6 kDa); trypsin inhibitor (20.1 kDa); and α-lactalbumin (14.2 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 using the Bio-Rad detergent compatible (DC) method (Bio-Rad Laboratories, CA), using BSA as standard.

2.13. Data analysis

Mathematical analysis of the data was performed by using the KINETIC, EBDA and LIGAND-PC curve-fitting programs [23]. Statistical comparisons for each of the binding and kinetic parameters with the control sample were made by using a paired Student's t-test. The data are expressed as the mean ± S.D. Statistical significance was reported if $P < 0.05$ was achieved.

3. Results

3.1. The LAGS is an integral membrane protein in the liver endoplasmic reticulum

LAGS' activity is mainly associated with rat liver endoplasmic reticulum (data not shown). Thus, we studied the possibility that DEX bound to an integral or peripheral membrane protein [18]. Treatment of microsomes

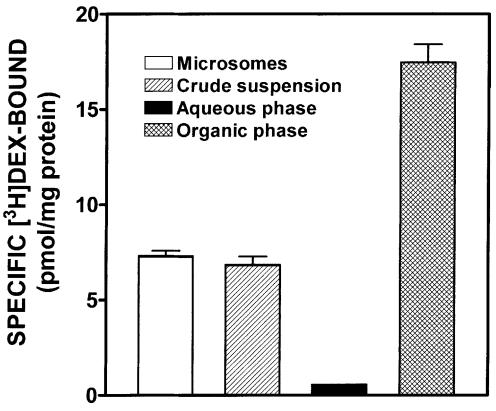

Fig. 1. Phase separation of LAGS from male rat liver microsomes. Microsomal suspensions (2–3 mg/ml) in the presence of 2 mM of Triton X-114 were submitted to phase separation, as described in Section 2. Aliquots of the crude microsome suspension, Triton X-114-solubilized microsomes, and of the aqueous and detergent phases were incubated with [^{3}H]DEX in the absence (total binding) and presence (non-specific binding) of a 200-fold excess of DEX, and analyzed for specific [^{3}H]DEX binding, as described in Section 2. The results represent the mean ± S.E.M. of three experiments.

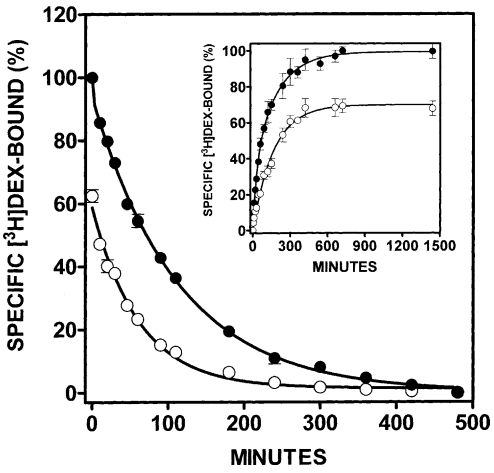

Fig. 2. Association and dissociation kinetics of specific [^{3}H]DEX binding to CHAPS-solubilized LAGS. The association time course (inset) was started with the addition of [^{3}H]DEX (150 nM) to 2 mg/ml solubilized proteins (●) in the absence (total binding) or in the presence (non-specific binding) of a 200-fold excess of unlabeled steroid. The incubation was continued at 0 °C until it reached equilibrium at 12 h. Then, the kinetic of dissociation was observed after the addition of unlabeled DEX to give a final concentration of 75 μM. The reactions were stopped by the addition of DCC to the incubation mixture at the times specified and the supernatant was counted as described in Section 2. Data obtained with 2 mg/ml crude microsomes (○) under the same conditions are included as reference. All data are expressed as a percentage of the maximal capacity, obtained after 12 h of incubation at 0 °C. Each point represents the mean ± S.E.M. of three separate experiments.

with 0.1–1 M KCl in TMMDSI did not give rise to the membrane-associated [^{3}H]DEX-binding activity. The protein content of the soluble fraction was maximum with 1 M KCl. To separate integral and peripheral proteins we used the procedure of phase partitioning with Triton X-114 [18]. Microsomes were first extracted with 0.75 M KCl and then collected by centrifugation and solubilized with Triton X-114, as described in Section 2. Optimal solubilization of [^{3}H]DEX-binding activity was achieved with a microsomal protein concentration of 2 mg/ml in 2 mM Triton X-114. During the subsequent phase, separation the major [^{3}H]DEX-binding activity was partitioned into the detergent phase (Fig. 1). These results show that [^{3}H]DEX-binding activity is associated with an integral membrane protein bound to the endoplasmic reticulum.

3.2. Pharmacological characterization of solubilized LAGS from rat liver microsomes

Preliminary experiments made it possible to establish conditions for optimal LAGS solubilization [24]. This was achieved with a microsomal protein concentration of 7 mg/ml with 7.5 mM CHAPS. Under these conditions, the detergent yielded almost 90% extraction of [^{3}H]DEX-binding activity, whereas 60% of total membrane proteins were solubilized.

To prove that the [^{3}H]DEX-binding site present in CHAPS-solubilized proteins represents the previously described LAGS [9–11], its properties were investigated in equilibrium binding studies. Specific binding to CHAPS-solubilized LAGS was time-dependent and reversible. As shown in Fig. 2 (inset), the interaction of [^{3}H]DEX with CHAPS-solubilized proteins was rapid, and 12 h of incubation were needed to reach the maximal binding capacity, which remained stable for at least 24 h (data not shown). In these experimental conditions, the association was best fitted following a monoexponential model (K_{+1} = 56000 ± 2565 min^{-1} M^{-1}). Once [^{3}H]DEX-binding to solubilized LAGS reached equilibrium, the dissociation kinetics of [^{3}H]DEX from LAGS was observed by the addition of 500-fold excess of an unlabeled steroid. 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 (Fig. 2). Specific [^{3}H]DEX binding to LAGS was reversible, and a monoexponential model explained DEX-induced dissociation (K_{-1} = 0.0060 ± 0.001 min^{-1}). The Scatchard plot of the equilibrium binding of [^{3}H]DEX to solubilized LAGS is linear, suggesting the labeling of a single class of binding sites with a K_d = 104 ± 2 nM and a B_{max} = 15 ± 1 pmol/mg of protein (Fig. 3). Corresponding values in membranes were K_d = 100 ± 6 nM and B_{max} = 13 ± 1 pmol/mg. No significant differences were observed between one- and two-site fits of data. The CHAPS-solubilized LAGS showed the same hierarchy of steroid affinities as those previously reported for the membrane-associated LAGS (data not shown) [9].

Cumulatively, these data show that [^{3}H]DEX binding to the CHAPS-solubilized LAGS is reversible, saturable, and exhibits all the pharmacological properties described for LAGS in male rat liver microsomes.

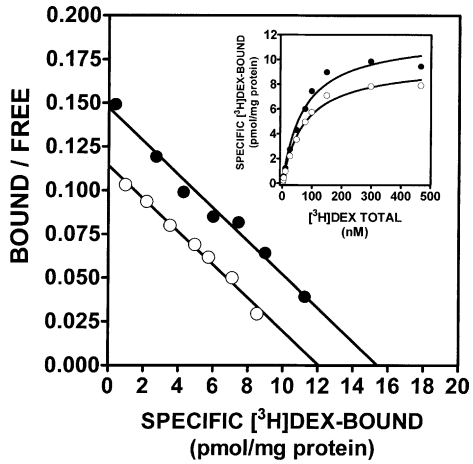

Fig. 3. Saturation binding of [^{3}H]DEX to CHAPS-solubilized LAGS. One hundred microliters of 2 mg/ml solubilized proteins (●) were incubated overnight with increasing concentrations of [^{3}H]DEX (final concentration, 5–500 nM), in the absence (total binding) or in the presence (non-specific binding) of a 200-fold excess of unlabeled DEX. The unbound steroid was removed by adsorption to DCC. B_{max} (15 ± 1 pmol/mg protein) and K_d (104 ± 2 nM) values were estimated by the non-linear least-square curve-fitting program LIGAND. Data obtained with 2 mg/ml crude microsomes (○) under the same conditions are included as reference. The data, representative of at least six independent experiments performed with similar results, are presented as the Scatchard plots and saturation curves (inset).

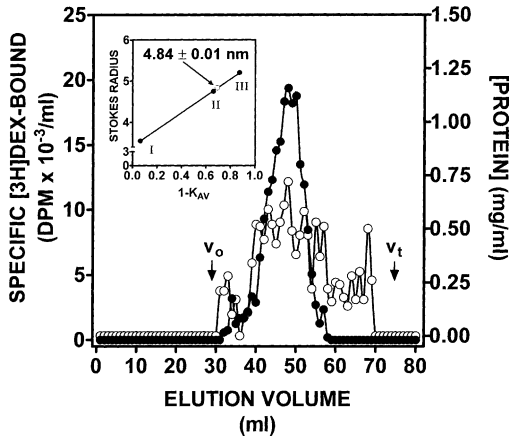

Fig. 4. Sephacryl S-300 chromatography of detergent-solubilized LAGS from rat liver. Aliquots (6–7 mg protein/ml) of CHAPS-solubilized LAGS were loaded on a Sephacryl S-300 column. The [^{3}H]DEX-binding activity (●) and protein content (○) were measured in each fraction (1 ml) as described in Section 2. The column was calibrated with proteins of known Stokes radius (inset). I, BSA; II, alcohol dehydrogenase; III, catalase. Results are representative of five experiments with similar results. V_0 and V_t indicate the void volume and total volume of the column, respectively.

3.3. Hydrodynamic characterization of the detergent solubilized LAGS

Since CHAPS proved to be the detergent of choice for optimal solubilization of LAGS, we studied the hydrodynamic properties of complexes formed by LAGS and this detergent. To determine their molecular size, CHAPS extract was chromatographed on a Sephacryl S-300 column. We used Ackers' method to construct the calibration graphs, getting a good linear relationship between Stokes radius and partition coefficient (Fig. 4, inset). Stokes radii of complexes were determined by interpolation in the calibration plots. [^{3}H]DEX-binding activity was found in one well-defined peak (Fig. 4). The Stokes radius of the [^{3}H]DEX-binding activity was 4.84 ± 0.02 nm in 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 from sucrose gradients made up in H_2O are shown in Fig. 5. A single peak of [^{3}H]DEX-binding activity was observed in the samples solubilized with CHAPS, and resolved in a 21–36% linear sucrose gradient containing 7.5 mM CHAPS (Fig. 5). It migrated with a $s_{20,w}$ of 6.5 S in H_2O (Fig. 5, inset). The $s_{20,w}$ of the LAGS–detergent complexes determined in H_2O was nearly the same as that 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.

The molecular mass of LAGS–detergent complexes were calculated from gel filtration and ultracentrifugation experiments. In the presence of the zwitterionic detergent CHAPS the complexes had a molecular mass of at least 134 kDa (Table 1).

3.4. Identification of the dexamethasone-binding peptides by photoaffinity labeling

To further characterize the glucocorticoid binding peptide(s) of LAGS, CHAPS-solubilized microsomes were irradiated in the presence of the glucocorticoid agonist [^{3}H]DEX in order to enable identification of the binding sites after SDS-PAGE. After removing excess [^{3}H]DEX using DCC, the samples were exposed to UV light for increasing periods of time. The maximal yield of photolabeling was achieved at 20 min. When photolabeled proteins were resuspended in SDS sample buffer containing 100 mM DTT followed by

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

Stokes radius (nm)	4.84 ± 0.02 (3)[a]
$s_{20,w}$ (S)	
In H_2O	6.50 ± 0.03 (3)
In D_2O	6.26 ± 0.42 (2)
s (S)[b]	6.383
Partial specific volume (ml/g)[c]	0.73
Molecular mass (kDa)[d]	134.2 (133.9–134.5)[e]

[a] Data represent mean ± S.D. (number of experiments).
[b] Data were calculated from all possible combinations of data obtained from gradients in H_2O and D_2O.
[c] It is assumed to be identical to that of standard proteins.
[d] See Section 2 for calculation.
[e] This range was calculated using S.E.M.

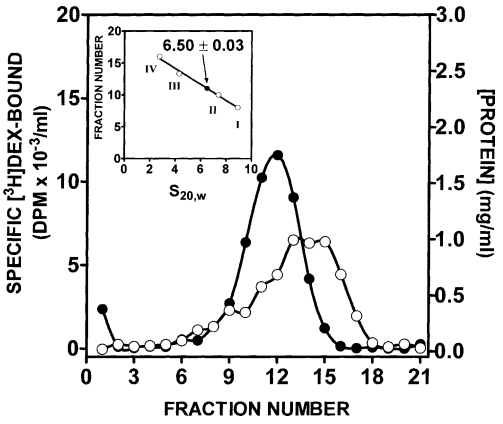

Fig. 5. Hydrodynamic characterization of the detergent-solubilized LAGS from rat liver. Aliquots (7–8 mg protein/ml) of CHAPS-solubilized LAGS were loaded onto 21–36% sucrose gradients and centrifuged at 350,000 × *g* for 16 h. Fractions (200 μl) were analyzed for [^{3}H]DEX-binding activity (●) and total protein concentration (○), as described in Section 2. Calibration plots [33] were constructed by ultracentrifuging proteins of known $s_{20,w}$ under the same conditions (inset) and used to estimate the $s_{20,w}$ value of the LAGS. Protein markers were: I, β-amylase; II, alcohol dehydrogenase; III, BSA; IV, carbonic anhydrase. Results are representative of five experiments with similar results.

SDS-PAGE, we detected two labeled peptides of 53 ± 4 and 37±2 kDa (Fig. 6). Interestingly, the [^{3}H]DEX-photolabeled peptides resuspended in sample buffer without DTT revealed the same specific labeling of 53 and 37 kDa (data not shown), thus suggesting that they are not disulfide-linked peptides. The above results together with these data seem to suggest the hypothesis that [^{3}H]DEX binds to an oligomeric protein, composed of at least two peptides of different size.

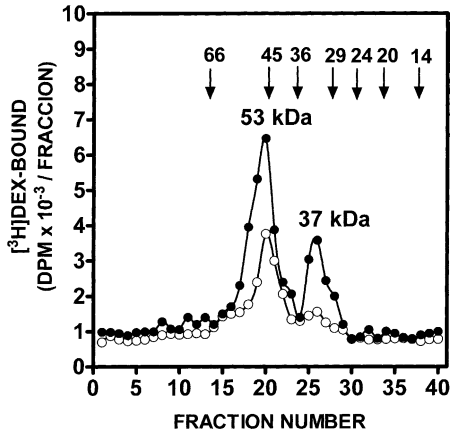

Fig. 6. SDS-PAGE analysis of [^{3}H]DEX-photoaffinity-labeled peptides. CHAPS-solubilized proteins from adult male rat liver microsomes were photoaffinity labeled with [^{3}H]DEX (400 nM) in the presence (○) or in the absence (●) of 60 μM of unlabeled DEX as described in Section 2. The covalent [^{3}H]DEX-binding proteins were concentrated with an Amicon YM filter and dissolved in sample loading buffer. Aliquots (1 mg of protein) were electrophoretically separated on a 10–20% polyacrylamide gel gradient under reducing conditions or under non-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 five experiments performed with similar results.

4. Discussion

The results presented above provide the basic tools for the further study of the structural and functional significance, in drug action and in normal cellular physiology, of specific glucocorticoid binding sites associated with an oligomeric protein in membranes from rat liver endoplasmic reticulum. This oligomeric protein carries specific binding sites for glucocorticoids, progestins, and 17α-alkylated steroids but not for the potent glucocorticoid agonist triamcinolone acetonide. Thus far, the evidence suggests that most of the steroids that this protein is capable of binding have powerful effects on the liver and that 17α-AA can interact and modulate the LAGS' activity (i.e. the glucocorticoid-binding activity) under physiological or pharmacological conditions.

The LAGS were solubilized in a functional form. The Scatchard plot of the [^{3}H]DEX binding to solubilized proteins revealed a higher B_{max} level in CHAPS extract than in membranes and a K_d value of LAGS similar in CHAPS extracts and in membranes. This suggests that modification of the phospholipid environment of LAGS does not affect protein-binding affinity. This feature has been observed for some binding proteins but not all [24]. The Scatchard plot yielded a straight line in the range from 10 to 500 nM, which indicates that solubilized LAGS is composed of a unique class of binding sites, as with membrane-bound LAGS [9]. This model is reinforced by the fact that both association and dissociation kinetics fit a monophasic interaction of [^{3}H]DEX with the LAGS (see Fig. 2).

Gel permeation chromatography of CHAPS extract showed that LAGS is solubilized in a single form with a Stokes radius of 4.84 nm (Fig. 4). Sucrose density gradient ultracentrifugation of CHAPS-solubilized LAGS also showed a single [^{3}H]DEX-binding component, with a sedimentation coefficient of 6.4 S (Table 1). These experiments did not show any high molecular mass forms of solubilized protein that would be eluted in the V_0 of the Sephacryl S-300 column or would be pelleted during ultracentrifugation. Thus, it appears that protein aggregation does not occur in our experimental conditions despite the fact that these hydrodynamic studies were carried out in the presence of detergent concentrations below their critical micellar concentration [24]. Since no significant difference was observed between the sedimentation coefficient values measured in H_2O and D_2O, we assume 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 [21]. Therefore, in making the calculations, a partial specific volume of 0.73 cm^3/g was used, as selected by Martin and Ames for their sucrose density centrifugation studies [33]. Taking the cumulative values obtained from hydrodynamic studies, the molecular mass of the LAGS was estimated to be at least 135 kDa.

SDS-PAGE of photoaffinity-labeled LAGS, both in reducing and non-reducing conditions, yielded molecular masses estimated for two [^{3}H]DEX-binding peptides of 53

and 37 kDa. It is possible that the 37 kDa peptide may result from the proteolytic cleavage of the 53 kDa peptide but the cocktail of proteolysis inhibitors used makes this unlikely. The reason why the 53 and 37 kDa polypeptides can be observed in photoaffinity labeling experiments but not in hydrodynamic experiments may be tentatively ascribed to the fact that LAGS can be cleaved, yet the fragments will remain associated because of strong hydrophobic interactions; these fragments can only be separated following denaturation in SDS. The molecular mass estimated for [^{3}H]DEX-binding protein identified by hydrodynamic studies (i.e. 134 kDa) cannot be obtained just by the sum of the molecular masses of the individual peptides identified by SDS-PAGE analysis after photoaffinity labeling of LAGS, i.e. 53 and 37 kDa (90 kDa). We could explain this discrepancy in two ways: (1) the peptides may form a dimeric structure in native conditions; and (2) we detect in SDS-PAGE only those peptides that are able to bind [^{3}H]DEX. These data suggest that [^{3}H]DEX-binding peptides are associated with an oligomeric protein with a molecular mass of at least 134 kDa.

Several steroid-binding sites associated with oligomeric proteins, distinct from nuclear receptors, have been reported in liver membranes: a DEX-mesylate binding site in rat endoplasmic reticulum containing two 45 kDa peptides [25], a progesterone binding protein containing two polypeptides of 28 and 56 kDa, respectively [26,27], a cortisol binding protein from hepatic plasma membrane with two polypeptides of 52 and 57 kDa, respectively [28], and a STBP in rat endoplasmic reticulum containing three polypeptides of 55, 31 and 22 kDa [4]. At present, we cannot completely discard the possibility that these steroid-binding peptides may be related to LAGS, but many findings make this unlikely [4,9,26].

The mechanism of interaction of DEX with LAGS can be clearly distinguished from the closest related candidate, STBP [4]. ST interaction with STBP suggests the existence of positive cooperativity [4], reinforced by the fact that both dissociation and association kinetics of ST to STBP are biphasic, indicating the existence of a complex interaction between ST and STBP [4]. This contrasts with the data described above for LAGS (Fig. 3). Moreover, STBP shows an extremely narrow pharmacological profile, being selective for ST and its analog, DA, among more than 100 steroidal and non-steroidal compounds assayed, including all those that are able to displace [^{3}H]DEX-binding activity [4]. In contrast to LAGS, SDS-PAGE of photoaffinity [^{3}H]ST-labeled peptides in reducing conditions yielded molecular mass estimates for three [^{3}H]ST-binding peptides of 55, 31 and 22 kDa [4]. However, unlike the LAGS, SDS-PAGE of photoaffinity [^{3}H]ST-labeled peptides under non-reducing conditions revealed a 104 kDa peptide, suggesting the existence of disulfide bonds present within their peptides [4]. These findings together with the findings that, unlike LAGS, STBP is already present at birth [4] and that LAGS has a half live shorter than STBP [8] suggest that these steroid-binding proteins are oligomeric in nature but different in peptidic composition.

Although there are many hypotheses that assign functional roles for microsomal binding sites, such as involvement in post-transcriptional actions of steroid hormones [2,3], our studies can clarify neither the biological role nor the mechanism of action of the LAGS. However, it is interesting that [^{3}H]DEX-binding activity is associated with an integral membrane protein, which negates the possibility that its binding protein could be a secretory protein. Since [^{3}H]DEX-binding activity is insensitive to cytochrome P450 inhibitors or sulfated steroids [9] and has an apparent molecular mass of 135 kDa, it is unlikely that LAGS is a steroid-metabolizing enzyme, such as the cytochrome P450 family [29] (48–56 kDa) or flavin monoxygenases [30] (55–59 kDa). In addition, members of a family of GTP-binding proteins recently identified in the endoplasmic reticulum membrane possess similar molecular masses (20–30 kDa) [31] to that of the 37 kDa [^{3}H]DEX-binding peptide. Some of them are also associated in larger structures (about 100 kDa) [32]. Thus, since a relationship between the 37 kDa polypeptide and these GTP-binding proteins cannot be excluded, we are currently investigating if [^{3}H]DEX binding to LAGS is modulated by GTP and GTP analogues.

In summary, we have report low-affinity glucocorticoid-binding peptides associated with an oligomeric membrane protein from endoplasmic reticulum. The inducibility of this protein [9–12] and its successful solubilization pave the way for large-scale solubilization and ultimate purification of this protein.

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