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MICROSOMAL DEXAMETHASONE BINDING SITES IDENTIFIED BY AFFINITY LABELLING

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Summary—Binding studies with [^{3}H]dexamethasone† identified a class of binding sites on male rat liver microsomes. The binding sites were glucocorticoid-dependent and specific for glucocorticoids and progestins. Scatchard binding parameters, competition studies with triamcinolone acetonide, a synthetic glucocorticoid which competes well for the glucocorticoid receptor, and immunoblotting with an antiglucocorticoid receptor antibody indicated that these sites are distinct from the cytosolic glucocorticoid receptor. Affinity labelling experiments with [^{3}H]dexamethasone 21-mesylate revealed two specifically labelled peptides, one at approx. 66 kDa and a doublet at 45 kDa. The 66 kDa peptide had been previously identified in serum and may be present as a result of serum contamination of the microsomal preparation. The 45 kDa doublet, on the other hand, had been shown to be absent from rat serum. The characteristics of the 45 kDa peptide(s) were identical to those of the dexamethasone binding site identified in the binding studies. [^{3}H]Dexamethasone binding characteristics and affinity labelling of microsomal subfractions, separated by isopycnic centrifugation, showed that the binding sites are located in the endoplasmic reticulum. The identification and role of the 45 kDa peptide doublet remain to be determined.

INTRODUCTION

The mechanism of action of steroid hormones and their regulation is of vital importance to our understanding of control of growth and development via altered cellular function. Although much knowledge exists about the molecular events associated with steroid effects on gene expression [1, 2] there is still very little known about several steps in the mechanism of steroid action [3]. Due to the basic lack of knowledge about steroid interactions with membrane binding components which may have possible transport or regulatory roles, we have been characterizing, identifying and isolating membrane binding components in order to investigate their roles in hormone action.

Glucocorticoid binding to microsomes is well documented [4–8]. For a general review of steroid binding to membranes refer to Szego and Pietras[9]. In light of some recent work in our laboratory [10] and others [11–13] demonstrating glucocorticoid binding to the plasma membrane, the demonstration of a glucocorticoid receptor-like antigen on lymphoma plasma membranes [14], and our demonstration of the presence of the glucocorticoid receptor on the nuclear envelope [15–17], we wished to investigate glucocorticoid interaction with microsomes, as the further identification and localization of the dexamethasone binding sites will shed light on the intracellular dynamics of the glucocorticoid hormone and its receptor. In this paper, we describe the characterization and localization of [^{3}H]dexamethasone binding to microsomes and microsomal subfractions in intact and adrenalectomized animals. In addition, we have succeeded in identifying the microsomal and microsomal subfraction dexamethasone binding sites with the affinity label [^{3}H]dexamethasone 21-mesylate.

EXPERIMENTAL

Animals

Adult male Sprague–Dawley rats weighing 225–250 g were obtained from Charles River, Canada and maintained on a diet of Purina Lab Chow and tap water *ad libitum*. The rats were sacrificed by decapitation and exsanguination. The livers were quickly removed and placed in two volumes of ice-cold homogenization buffer [0.25 M sucrose, 25 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM Tris–HCl, pH 7.6]. Adrenalectomy under ether anaesthesia was performed by Charles River Laboratories. Adrenalectomized animals subsequently received 0.9% NaCl in their drinking water. They were used for experiments 7–9 days after adrenalectomy.

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†*Abbreviations:* Dexamethasone; 9α-fluro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione: Dexamethasone 21-mesylate; dexamethasone 21-methylsulfonate: Triamcinolone acetonide; 9α-fluoro-11β,16α, 17α, 21 - tetrahydroxypregna - 1, 4 -diene 3,20-dione 16, 17acetonide: IgG; gamma immunoglobulin: M_r; relative molecular weight.

Microsome preparation

Microsomes were prepared after differential centrifugation by the method of Omrani *et al.*[4] with the following modifications: (1) substitution of DTT for β-mercaptoethanol in the homogenization buffer, and (2) the addition of a final wash i.e. resuspension of microsomes in homogenization buffer or TAPS–NH_4OH, pH 8.6, and centrifugation at 51,520 *g* for 30 min before final resuspension in the appropriate buffer.

Microsomal subfractionation procedure

The procedure was a modification of a method applied to bovine anterior pituitary microsomes [18]. Microsomes from approx. 2 g starting material in homogenization buffer (density = 1.03 g/ml) were layered on top of a discontinuous sucrose gradient in a 38.5 ml ultracentrifuge tube. The gradient consisted of 5 ml homogenization buffer overlayered on 10 ml of each of 32, 40 and 50% (w/w) sucrose giving layers of defined densities of 1.14, 1.18 and 1.23 g/ml, respectively. Gradients were centrifuged for 14–16 h at 4°C at 80,000 g_{av} in a Beckman SW28 rotor. Membrane bands were collected and diluted to 8–10% sucrose with homogenization buffer lacking sucrose, and were centrifuged at 80,000 g_{av} for 1 h. Pellets were resuspended in either homogenization buffer without DTT at pH 7.6 or in TAPS at pH 8.6 for binding assays. Fractions were sometimes frozen in liquid nitrogen for up to 5 days, with no loss of binding and were thawed on ice prior to binding assays.

Protein determination and enzyme assays

The protein content was determined by the method of Lowry *et al.*[19], using BSA as standard. 5'-Nucleotidase (EC 3.1.3.5) was assayed by the method of Goldfine *et al.*[20]. The P_i liberated was quantified by the method of Ames[21] using disposable phosphate-free glassware. Glucose-6-phosphatase (EC 3.1.3.9) was assayed by the method of Swanson[22] and P_i released assayed [21].

Binding assays

Membrane suspensions containing 100–200 μg protein were incubated in triplicate at 0–4°C with [^{3}H]dexamethasone in a total volume of 250 μl of homogenization buffer, pH 7.6, containing 1% ethanol without DTT, or TAPS, pH 8.6, containing 1% ethanol, with or without a 1000-fold excess of unlabelled dexamethasone or other unlabelled steroid. Binding was stopped by adding 1 ml of ice-cold buffer, containing 1% ethanol but no DTT, and separation of the bound from the free steroid by draining the incubation tubes onto presoaked GF/C filters (Whatman) placed on a vacuum manifold (Millipore). Filters were rinsed four times with 2 ml of the buffer and placed in the bottom of 20 ml glass scintillation vials. Counts were solubilized with 500 μl of NCS tissue solubilizer (Amersham) by incubating at room temperature overnight. To each vial was added 10 ml of Ready-Protein scintillation fluid (Beckman) and the vials were left in the dark overnight before radioactivity was determined by scintillation counting on a Beckman LS3801.

Affinity labelling

Microsomes and subfractions (200 μg/tube) were incubated with 220 nM [^{3}H]dexamethasone 21-mesylate for 6 h at 4°C in 250 μl of 25 mM TAPS, pH 8.6. The incubation was terminated by the addition of 750 μl ice-cold buffer and centrifugation for 3 min at 356,000 g_{av} in a Beckman TL-A ultracentrifuge. The pellet was washed two more times and finally resuspended in SDS-sample buffer and heat denatured. Samples were run on SDS–PAGE using 7.5% running gels, and stained to allow scanning on an LKB Ultroscan XL Enhanced Laser Densitometer. The gels were cut into 2 mm slices, dissolved in 150 μl 50% H_2O_2:0.8% NH_4OH (19:1, v/v) by heating to 70°C for several hours [23]. The H_2O_2 was neutralized with catalase before scintillation counting.

Gel electrophoresis and western analysis

SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) was performed using the discontinuous buffer system of Laemmli[24]. Vertical slab gels (18 × 16 × 0.15 cm) consisted of a 3.9% stacking gel and a 7.5% running gel. Samples in SDS-sample buffer [63 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS (w/v), 0.05% β-mercaptoethanol (v/v) and 0.0013% bromophenol blue (w/v)] were denatured by heating for 5 min at 96°C. Samples, denatured in SDS-sample buffer, were sometimes stored at −20°C. Samples were run at 30 mA/gel.

Gels were stained for 1 h in a solution of 0.2% Coomassie Brilliant Blue R-250, 7% acetic acid and 50% methanol and destained with 2–3 changes of a solution of 7% acetic acid and 50% methanol followed by several changes of a solution of 7% acetic acid and 5% methanol with gentle shaking.

Proteins for immunoblotting were electrophoresed on a 7.5% gel along with cytosol prepared from adrenalectomized animals [25], as a positive control for staining of the glucocorticoid receptor with the monoclonal antibody M7 [26]. Proteins were transferred electrophoretically onto nitrocellulose filters based on the procedure by Towbin *et al.*[27]. After incubating with a 1:400 dilution of the primary antibody, the blot was incubated with horseradish peroxidase conjugated goat anti-mouse IgG (1:2000). The blot was developed with a reagent containing 4-chloro-1-naphthol in the presence of 0.015% H_2O_2.

Chemicals

[6,7(*N*)-^{3}H]Dexamethasone (49.9 Ci/mmol and [6,7(*N*)-^{3}H]dexamethasone 21-mesylate (49.9 Ci/mmol) were purchased from NEN-Dupont. Un-

labelled steroids were from Steraloids Inc. except for cholesterol and triamcinolone acetonide, which were from Sigma Chemical Company. Sucrose (Ultra-pure) was from Schwartz-Mann (ICN). TAPS (*N*-tris-[hydroxymethyl] methyl-3-amino propane-sulfonic acid) and Trizma base were from Sigma. All chemicals for polyacrylamide gel electrophoresis and Western blotting were from BioRad Laboratories. BSA (bovine serum albumin, fraction V) was purchased from Sigma. All other chemicals were of reagent grade or better.

RESULTS

Characterization of microsomal dexamethasone binding sites

We first determined equilibrium conditions for the binding of [^{3}H]dexamethasone to male rat liver microsomes. Our previous studies of dexamethasone interactions with the rat liver plasma membrane had shown that considerable degradation of dexamethasone binding sites occurred at 22 and 37°C [10]. Therefore, time-course studies of the microsomal fraction were carried out at 0–4°C to reduce thermolability. Incubations were in TAPS buffer, pH 8.6, as we had established in our previous affinity labelling studies with dexamethasone 21-mesylate [10, 16] that this buffer gave optimal results. Furthermore, our preliminary binding studies showed that there was no difference in numbers of binding sites when the incubations were carried out in homogenization buffer at pH 7.6 or in TAPS buffer at pH 8.6 (results not shown). Maximum binding had been attained at 8 h and remained stable to 16 h, thereafter binding declined (Fig. 1). Therefore, equilibrium conditions for the binding of [^{3}H]dexamethasone to rat liver microsomes were 8–10 h incubations at 0–4°C.

Specific binding of [^{3}H]dexamethasone to rat liver microsomes demonstrated saturability (Fig. 2A) and hence was subjected to Scatchard analysis (Fig. 2B). The analysis revealed a single class of binding sites with a K_d of 68 nM and a B_{max} of 23 pmol/mg protein.

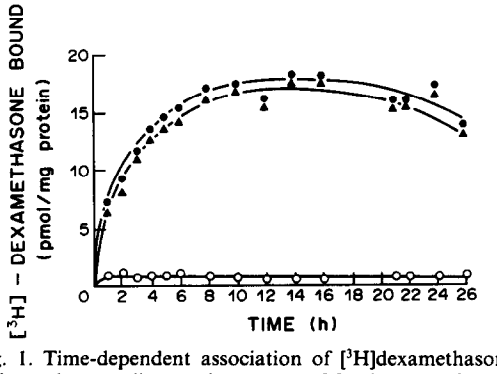

Fig. 1. Time-dependent association of [^{3}H]dexamethasone with male rat liver microsomes. Membranes (200 μg protein/tube) were incubated in triplicate with 50 nM [^{3}H]dexamethasone in the presence or absence of 50 μM unlabelled dexamethasone at 0–4°C. Specific binding (▲) for each time point was calculated from the difference of [^{3}H]dexamethasone binding in the presence (○, non-specific) and absence (●, total binding) of excess unlabelled dexamethasone.

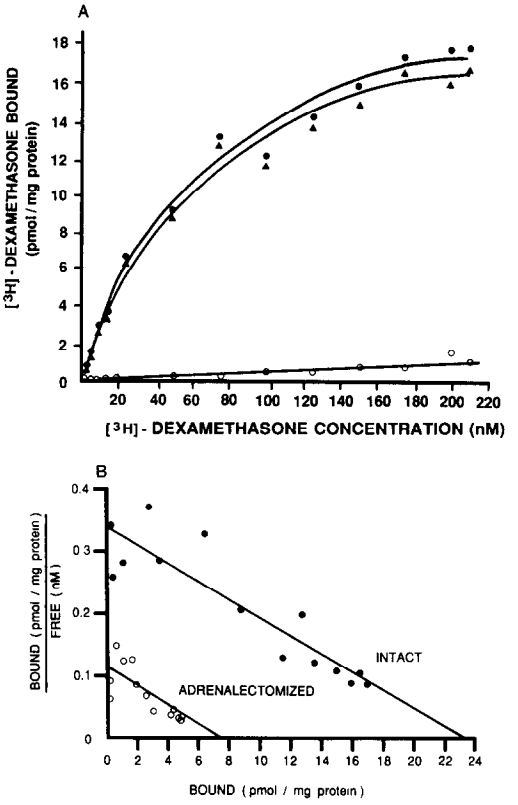

Fig. 2. Analysis of [^{3}H]dexamethasone binding to rat liver microsomes. Rat liver microsomes (200 μg protein) were incubated with [^{3}H]dexamethasone (1–210 nM) in the presence or absence of a 1000-fold excess of unlabelled dexamethasone for 10 h at 0–4°C. (A) Saturation curve of binding to liver microsomes from intact rats. Specific binding (▲) was determined by subtraction of non-specific binding (○) from total binding (●). (B) Scatchard analysis [35] of specific [^{3}H]dexamethasone binding to liver microsomes obtained from intact rats (●) and from adrenalectomized rats (○) analyzed by linear regression analysis.

Table 1. Binding parameters of [^{3}H]dexamethasone binding to male rat liver microsomes and subfractions

	Fraction	K_d	B_{max}	r
Intact animal	M	68.2	23.1	−0.905
	P1	61.0	18.6	−0.855
	P2	121.0	56.4	−0.681
	P3	89.2	28.4	−0.865
Adrenalectomized animal	M	57.6	6.7	−0.780
	P1	52.1	4.2	−0.844
	P2	46.9	15.2	−0.979
	P3	82.6	13.1	−0.791

Rat liver microsomes and subfractions (200 μg protein/tube) from intact or adrenalectomized male rats were incubated (in triplicate) at 0–4°C with [^{3}H]dexamethasone (1.0–210 nM) in the presence or absence of a 1000-fold excess of unlabelled dexamethasone for 8–10 h in 250 μl TAPS pH 8.6 containing 1% ethanol. Specific binding was determined by subtracting non-specific binding i.e. tubes with a 1000-fold excess of unlabelled dexamethasone from total binding i.e. tubes without excess unlabelled dexamethasone. Scatchard plots were analyzed by linear regression analysis and the dissociation constant (K_d), binding capacity (B_{max}) and correlation coefficient (r) calculated.

Table 2. Specificity of [^{3}H]dexamethasone binding to male rat liver microsomes and subfractions

Unlabelled hormone	% Inhibition of total binding			
	M	P1	P2	P3
Dexamethasone	98.3	96.3	98.0	96.2
Triamcinolone acetonide	14.1	17.4	34.5	12.1
Cortisol	98.6	98.3	97.7	97.8
Corticosterone	98.7	96.4	98.2	97.5
Deoxycorticosterone	98.3	97.8	98.0	94.6
Progesterone	98.7	98.3	98.2	96.9
Estradiol-17β	90.5	91.6	91.6	ND
Testosterone	82.0	84.4	83.7	79.1
Cholesterol	0	7.0	11.2	7.9

Rat liver microsomes and subfractions (100 μg protein/tube) were incubated with 40 nM [^{3}H]dexamethasone for 10 h at 0–4°C in the presence of 8, 16 and 40 μM (200-, 400- and 1000-fold excess) of the unlabelled steroid. The data presented here are expressed as % inhibition of total binding by 1000-fold excess of unlabelled competitor.
ND = not determined.

Analysis of binding to microsomes obtained from animals which had been adrenalectomized showed that there is no change in the affinity of these sites upon glucocorticoid depletion, but there is an approx. 75% decrease in the binding capacity (Fig. 2B and Table 1).

The steroid specificity of the microsomal [^{3}H]dexamethasone binding sites was assessed by competition analysis with unlabelled steroids (Table 2). The natural glucocorticoids corticosterone, deoxycorticosterone and cortisol, competed for the [^{3}H]dexamethasone binding sites with the same potency as unlabelled dexamethasone. The glucocorticoid analogue, triamcinolone acetonide, which competes well for the binding of dexamethasone to the glucocorticoid receptor [28], was a relatively poor competitor for these sites. The progestin, progesterone, competed as effectively as dexamethasone while the estrogen, 17β-estradiol, was a poorer competitor and the androgen, testosterone, was an even poorer competitor. Cholesterol, the steroid precursor, did not compete for the binding of [^{3}H]dexamethasone to the rat liver microsomes.

Identification of microsomal dexamethasone binding sites by affinity labelling

We next affinity labelled microsomes with the anti-glucocorticoid [^{3}H]dexamethasone 21-mesylate [29] in order to enable identification of the binding sites after SDS–PAGE. As mentioned above, affinity labelling was greater at pH 8.6 than at pH 7.4 for both plasma membrane and nuclear envelope [10, 16]. Therefore, microsomes were also labelled at pH 8.6. Figure 3A shows the radioactivity profile of an SDS–polyacrylamide gel of microsomes after affinity labelling with [^{3}H]dexamethasone 21-mesylate. The profile clearly shows two major specifically labelled peaks, one at approx. 45 kDa which sometimes appeared as a doublet and one at approx. 66 kDa. In one experiment, protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin A and 10 U/ml aprotinin) were added to

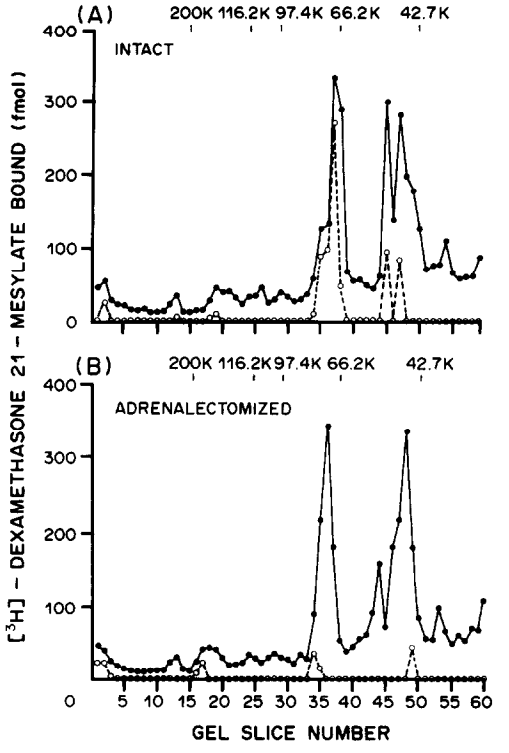

Fig. 3. Affinity labelling of male rat liver microsomes with [^{3}H]dexamethasone 21-mesylate. Membranes (200 μg protein) were incubated with 220 nM [^{3}H]dexamethasone 21-mesylate for 6 h at 0–4°C in 25 mM TAPS–NH_4OH, pH 8.6 to determine total binding. Non-specific binding was determined by the addition of [^{3}H]dexamethasone 21-mesylate to membranes which had been incubated with 220 μM unlabelled dexamethasone for 6 h and subtracted from total binding to give specific binding. The affinity labelled proteins were heat-denatured in SDS-sample buffer and run on 7.5% SDS–PAGE. The gel was cut into 2 mm slices and dissolved in 150 μl of 50% hydrogen peroxide–0.8% ammonia (19:1, v/v) at 60–70°C. After the addition of 10^3 U of catalase and 25 mg of ascorbic acid in a total volume of 500 μl, the sample was counted in 10 ml of Ready-Protein. Total binding (●) and specific binding (○) are shown. Molecular weight standards are myosin (200,000 Da), β-galactosidase (116,250 Da), phosphorylase B (97,400 Da), bovine serum albumin (66,200 Da) and ovalbumin (42,699 Da).

buffers used in the preparation of the microsomes. The peptide labelling pattern was not altered by the protease inhibitors, suggesting that the affinity labelled peptides are not degradation products of higher molecular weight peptides. Next we examined the profiles of affinity labelled microsomes obtained from adrenalectomized animals (Fig. 3B). This figure shows that both specifically labelled entities were reduced upon adrenalectomy, indicating a dependency on glucocorticoids. We had previously shown the presence of the 66 kDa entity in serum [10] and thus suspect that it is in the microsomal preparation at least in part as a result of serum contamination. Furthermore, its response to adrenalectomy was variable; in some experiments the specifically labelled band at 66 kDa was not reduced by adrenalectomy.

The 45 kDa entity, on the other hand, was not present in serum and its response to adrenalectomy was very reproducible. Thus, this peptide doublet resembled the site identified in the [^{3}H]dexamethasone binding studies. The 66 kDa site may be a site which is affinity labelled by dexamethasone 21-mesylate, but which is not a dexamethasone binding site. A likely candidate for this entity is serum albumin.

Sublocalization of microsomal dexamethasone binding sites

In order to further localize the dexamethasone binding sites, microsomes were first subfractionated on a discontinuous sucrose density gradient. Then [^{3}H]dexamethasone binding to the subfractions was characterized followed by identification of polypeptides affinity labeled with [^{3}H]dexamethasone 21-mesylate.

The subfractionation procedure yielded 4 subfractions: particulate material obtained at the 1.03/1.14 g/ml interface (P1), the 1.14/1.18 g/ml interface (P2), the 1.18/1.23 g/ml interface (P3) and the pellet (P4). Binding studies with the subfractions showed that the affinities for the [^{3}H]dexamethasone (Table 1), the responses to adrenalectomy (Table 1) and the steroid specificities as determined by competition analysis (Table 2) of the microsomal subfractions and the microsomes were similar. Subfractions were then affinity labelled with [^{3}H]dexamethasone 21-mesylate. The radioactivity profile obtained from an SDS–PAGE of each microsomal subfraction resembled the labelling of the microsomal fraction in that two specifically labelled peaks were seen, one at 66 kDa and a doublet at 45 kDa. As an example, the radioactivity profile of microsomal subfraction P2 after affinity labelling is shown in Fig. 4A. The affinity labelling pattern obtained after adrenalectomy of the animals is shown in Fig. 4B and is seen to resemble closely that obtained from microsomes of adrenalectomized animals.

Figure 5A shows that the plasma membrane was recovered predominantly in the lighter fractions, as the 5′-nucleotidase specific activity was highest in P1 and decreased as the density of the subfraction increased. The endoplasmic reticulum, as monitored with glucose-6-phosphatase, was found in all subfractions; however, the glucose-6-phosphatase specific activity was highest in P2 (Fig. 5B). As the profiles of enrichment of the two marker enzymes were distinct, we concluded that this subfractionation would allow us to determine whether dexamethasone binding sites were restricted to either the plasma membrane or to the endoplasmic reticulum.

Figure 5C shows that P2, which contained the greatest enrichment of glucose-6-phosphatase possessed the greatest number of [^{3}H]dexamethasone binding sites/mg membrane protein as deduced from Scatchard analysis (Table 1). As in the [^{3}H]dexamethasone binding studies, there is a correlation of the glucose-6-phosphatase enrichment with the enrichment of the binding of the 45 kDa site/mg protein in the various microsomal subfractions, as determined with the affinity labelling reagent, dexamethasone 21-mesylate (Fig. 5D).

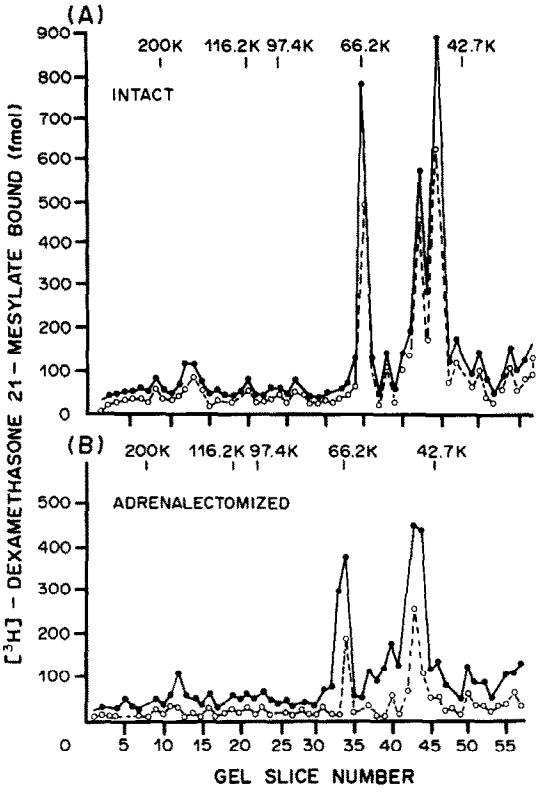

Fig. 4. Affinity labelling of male rat liver microsomal subfraction P2 with [^{3}H]dexamethasone 21-mesylate. Membranes (200 μg protein) were incubated with 220 nM [^{3}H]dexamethasone 21-mesylate for 6 h at 0–4°C in 25 mM TAPS–NH_4OH, pH 8.6 to determine total binding. Non-specific binding was determined by the addition of [^{3}H]dexamethasone to membranes which had been incubated with 220 μM unlabelled dexamethasone for 6 h and subtracted from total binding to give specific binding. The affinity labelled proteins were heat-denatured in SDS-sample buffer and run on 7.5% SDS–PAGE. The gel was cut into 2 mm slices and dissolved in 150 μl of 50% hydrogen peroxide–0.8% ammonia (19:1, v/v) at 60–70°C. After the addition of 10^3 U of catalase and 25 mg of ascorbic acid in a total volume of 500 μl, the sample was counted in 10 ml of Ready-Protein. Total binding (●) and specific binding (○) are shown.

Immunoblotting of the affinity-labelled peptides

As a possible candidate for a glucocorticoid binding site is the glucocorticoid receptor, a Western blot analysis of microsomal proteins with the anti-glucocorticoid receptor antibody, M7 [26] was obtained. While rat liver cytosol enriched in untransformed glucocorticoid receptor gave a positive result as expected, the immunoblotting of the rat liver microsomes was negative (results not shown). Molecular weights for actin have been reported which range from 42 to 48 kDa. We, therefore, also investigated whether the 45 kDa entity might be an actin isoform.

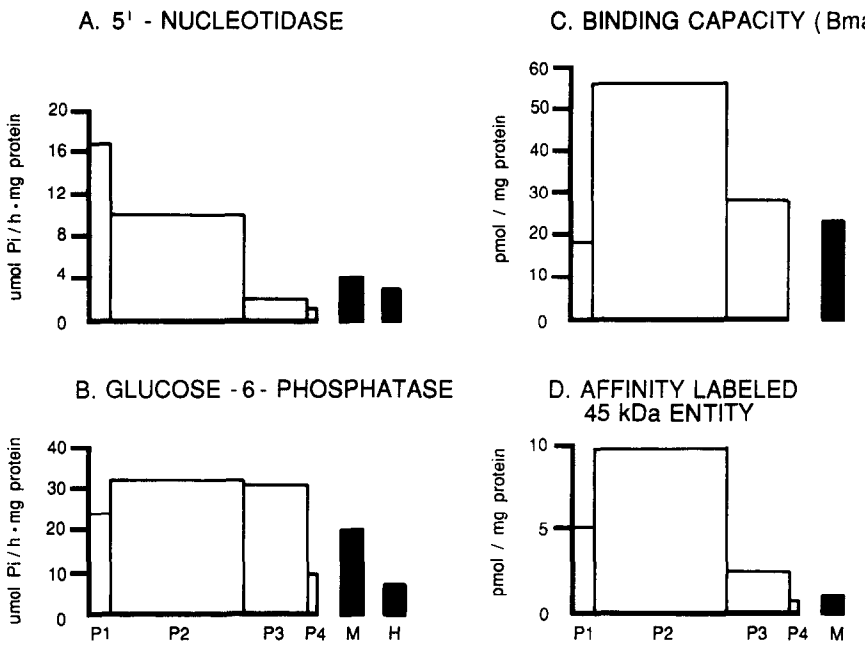

Fig. 5. Discontinuous sucrose density gradient fractionation of rat liver microsomes. Particulate material was recovered at density interfaces 1.03/1.14 (P1), 1.14/1.18 (P2), 1.18/1.23 (P3) and pellet (P4). Values are also reported for the homogenate (H) and the microsomes (M). The horizontal axis indicates the relative protein content of the individual fractions as a proportion of total microsomal protein. Values are expressed as the means of triplicate determinations which differed from the mean by ±10%.

However, while the anti-actin antibody identified a cross-reacting species, the antigen ran at 42 kDa [30]. There was no cross-reactivity in the region of the dexamethasone 21-mesylate binding, i.e. 45 kDa, and a doublet was not observed.

DISCUSSION

Scatchard analysis of [^{3}H]dexamethasone binding to male rat liver microsomes and microsomal subfractions identified one class of dexamethasone binding sites. Omrani *et al.*[4] and Ambellan *et al.*[5] have also reported a class of dexamethasone binding sites on rat liver microsomes having a similar affinity and binding capacity. We have previously reported the presence of dexamethasone binding sites in plasma membranes [10] and nuclear envelopes [16] from rat liver. Both of those membrane systems, however, possessed two classes of dexamethasone binding sites, one class displaying an affinity similar to the site reported here and one class displaying a higher affinity. It is possible that a higher affinity lower capacity site is present on the microsomes but is masked by the higher capacity site in this membrane fraction and is not detected with our methods.

The dexamethasone binding sites exhibited a broad range of steroid specificity in contrast to that reported for cytosolic glucocorticoid receptor [31]. Plasma membrane [10, 12] and nuclear envelope [16] dexamethasone binding sites have been characterized which show similar steroid specificity to the site described here. Further, the broad specificity of membrane-associated steroid binding proteins is not unique to glucocorticoid systems as such sites have been reported for estrogen on anterior pituitary microsomes [32] and tri-iodothyronine on rat liver nuclear envelopes [33]. Adrenalectomy did not affect the affinity of these sites but resulted in a marked decrease in the number of binding sites. The decrease in number of binding sites is greater than any differences due to differences in weight and age between intact and adrenalectomized rats [4].

Affinity labelling of rat liver microsomes with the electrophilic reagent [^{3}H]dexamethasone 21-mesylate identified two specifically labelled polypeptides, one at 66 kDa and a doublet at 45 kDa. The 45 kDa site responds to adrenalectomy in the same way as the [^{3}H]dexamethasone binding sites and, therefore, probably is identifying the dexamethasone binding sites. A possibility is that the 45 kDa doublet results from degradation of the glucocorticoid receptor. However, immunoblotting with an antiglucocorticoid receptor antibody did not detect the presence of the glucocorticoid receptor. Triamcinolone acetonide competes effectively with dexamethasone for the cytosolic glucocorticoid receptor [28] whereas triamcinolone acetonide was a poor competitor in these studies. These findings suggest that these sites are not the glucocorticoid receptor. However, the 45 kDa entity may be a fragment of the glucocorticoid receptor, which has lost the immunoreactive site by degradation. This possibility must be considered as the immunogenic domain of the rat glucocorticoid

receptor (amino acids 1-410/414) [26] is distinct from the site labelled with dexamethasone mesylate (cys 656) [34]. We have also considered the possibility that the 45 kDa peptide is actin, because of its similar molecular weight, Mg^{2+} dependence (results not shown), membrane association, abundance and putative role in transport. We were unable, however, to detect a 45 kDa microsomal peptide which cross-reacted with anti-actin antibody. Corticosteroid-binding globulin (transcortin) is another candidate, although this is less likely because dexamethasone does not bind serum globulins and affinity labelling of rat serum with [^{3}H]dexamethasone 21-mesylate did not label a 45 kDa entity [10]. A fourth possible identity for the 45 kDa peptide is that it is a steroid-metabolizing enzyme, although dexamethasone is not itself metabolized.

The 66 kDa site, on the other hand, was not altered consistently by adrenalectomy. Rat serum albumin has a M_r of 66 kDa and Simons has shown that, although [^{3}H]dexamethasone does not bind to serum albumin, [^{3}H]dexamethasone 21-mesylate labels serum albumin [34]. We have shown that affinity labelling of ammonium sulfate-precipitated serum resulted in a heavily labelled polypeptide of 66 kDa [10]. Therefore, the 66 kDa entity is probably rat serum albumin. Its presence in the microsomal preparation could be a result of serum contamination and of its synthesis in the liver. The fact that it is being contributed by two different pools would make variations in data more likely. We have previously shown that the 45 kDa sites are located in the plasma membrane [10], but the microsomal subfractionation studies presented here show that these sites are not localized solely to the plasma membrane. They are also present in the endoplasmic reticulum. We did not identify which subfractions contained the rough and smooth endoplasmic reticulum, although we would expect more rough membranes in the heavier fractions. Ambellan *et al.*[5] suggested a rough endoplasmic reticulum localization. However, they did not measure the plasma membrane contamination. Recently, Quelle *et al.*[12] suggest a correlation of increased dexamethasone binding with the enrichment of the plasma membrane marker. The many differences in their experimental design make comparisons with our results difficult, but their procedure in which fractions enriched in glycoproteins were prepared would favour plasma membrane purification.

In conclusion, we have identified a glucocorticoid-dependent rat liver microsomal peptide of 45 kDa which binds glucocorticoids. We have previously identified this binding site on the purified plasma membrane preparations [10]. In this study, we show that it is also present in the endoplasmic reticulum. The peptide may have a regulatory or transport role in steroid hormone action. Ultimate proof of its role in transport will come from the purification of this polypeptide and reconstitution into liposomes for transport studies.

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