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PHYSICOCHEMICAL ANALYSIS OF REVERSIBLE MOLYBDATE EFFECTS ON DIFFERENT MOLECULAR FORMS OF GLUCOCORTICOID RECEPTOR

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SUMMARY: Several distinct molecular forms of glucocorticoid receptor have been identified in a melanoma model system. We have used velocity sedimentation to monitor molybdate dependent alterations in receptor size and heterogeneity. In the absence of molybdate, native glucocorticoid receptor from dexamethasone-sensitive tumors sediments at 7-8 S and 12-13 S. Under identical conditions, receptor isolated from dexamethasone-resistant tumors sediments at 7-8 S only. However, when molybdate is introduced, either during homogenization or immediately prior to centrifugation, glucocorticoid receptors from both dexamethasone-sensitive and -resistant tumors sediment sharply at 9-10 S. These molybdate induced phenomena are reversible. The activated forms of glucocorticoid receptor isolated from both dexamethasone-sensitive and -resistant tumors by DEAE-cellulose chromatography have similar sedimentation coefficients (4-5 S) which are unaffected by molybdate.

INTRODUCTION

Steroid receptor proteins, as a class, have been difficult to study because of their extreme lability. This is particularly true when these proteins exist in their native, unliganded form (1-4). In addition, binding of steroid to receptor in cytosol preparations enables a variety of steroid dependent receptor alterations to take place (4). Because of our interest in understanding the detailed mechanism(s) of receptor mediated steroid hormone action, we wish to isolate the unaltered, native form of receptor so that we can study steroid binding and subsequent transformation of the complex to a nuclear binding form ("activation") in vitro.

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The initial discovery in Pratt's laboratory (5) that molybdate stabilized the glucocorticoid-receptor complex was quickly extended by several investigators. Now, virtually every class of steroid receptor protein has been shown to be stabilized by molybdate against degradation and/or activation in vitro (1-3,6-8). It also appears that molybdate can be a powerful tool to stabilize the native form of steroid receptor proteins during their isolation (8,9). This is particularly true for the glucocorticoid receptor (7,10-12). However, the mechanism and full potential of the molybdate induced stabilization is not yet understood (11). Indeed, there are still questions surrounding the description of molybdate induced physical alterations in the glucocorticoid receptor (1,10,13). In this paper we describe, for the first time, the use of molybdate to reversibly control pronounced alterations in the size and heterogeneity of glucocorticoid receptor proteins from a Syrian hamster melanoma model system.

Experimental Procedures

Materials: 6,7-[^{3}H]dexamethasone (50 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.). Purified bovine liver catalase (11.3 S), human gamma globulin (7.1 S), bovine serum albumin (4.4 S) bovine hemoglobin (4.4 S) and equine heart cytochrome c (1.95 S) were all obtained from Sigma Chemical Co. (St. Louis, Mo.) DEAE-cellulose (DE-52) was from Whatman, Inc. (Clifton, N.J.). Sephadex G-25 (coarse) was obtained from Pharmacia Fine Chemicals (Piscataway, N.J.). All other reagents were of analytical reagent grade.

Methods: The recent isolation and characterization of glucocorticoid-resistant (clone 5) and -sensitive (clone 6) clonal variants of the RPMI 3460 Syrian hamster melanoma cell line (14) are described elsewhere (15). RPMI 3460, clone 5 and clone 6 cells were grown as solid tumors in the dorsal flank region of 4-6 week old female Syrian hamsters. The tumors were harvested after 11-15 days of growth, cleaned and stored in liquid nitrogen until use. A complete description of these procedures as well as confirmation of tumor sensitivity to glucocorticoids is to appear elsewhere (16).

Cytosol was prepared and labeled with 20nM [^{3}H]dexamethasone overnight at 0°C as described previously (17) using TDG_{25} buffer (10mM Tris-HCl, 1.0mM dithiothreitol and 25% glycerol (by volume), pH 7.4 at 0°C) both without or with 10mM sodium molybdate ($TDG_{25}M$).

The activated form of the glucocorticoid-receptor complex from these tumors was isolated by DEAE-cellulose chromatography as described elsewhere (18).

[^{3}H]dexamethasone labeled cytosol or partially purified receptor fractions were passed through small (10-11ml) Sephadex G-25 columns to remove free steroid. Two hundred microliters of these fractions were then layered onto linear 5-20% sucrose density gradients prepared with TDG_{25} or $TDG_{25}M$ buffer (see figure legends). The gradients were centrifuged in an SW 60 Ti rotor at 60,000 rpm for 16 hr at 2°C using a Beckman L5-65 ultracentrifuge. Fractionation of gradients (from the bottom) into 0.1ml aliquots was accomplished using a Beckman universal fraction collector. Radioactivity was mesured (38% efficiency) in a Beckman model 3150T scintillation counter. Additionally, the bottom of each centrifuge tube was excised, extracted with ethanol and analyzed for tritium to detect any pelleted [^{3}H]dexamethasone-receptor complexes.

The linearity of representative gradients was confirmed for each set of experimental conditions by refractometry and by optical density after addition of bromphenol blue to the limiting solution in the gradient forming device. Peaks of [^{3}H]dexamethasone binding activity were assigned sedimentation coefficients ($s_{20,w}$) according to the method of Martin and Ames (19). The sedimentation pattern of external markers[1] was established by their absorbance at 280nm. The correlation coefficient of the linear relationship between migration distance and $s_{20,w}$ of the marker proteins was routinely better than 0.996.

Scatchard analyses and sucrose density gradient centrifugation have been used to demonstrate that cytosols prepared from the melanoma tumors described in this communication contain no nonsuppressible [^{3}H]dexamethasone binding sites (16).

Results

In the absence of molybdate (TDG_{25} buffer), [^{3}H]dexamethasone-receptor complexes present in cytosol prepared from clone 5 (glucocorticoid-resistant) tumors sediment at 7-8 S. A representative profile is shown in Figure 1A. Under identical conditions, however, cytosolic [^{3}H]dexamethasone-receptor complexes derived from clone 6 (glucocorticoid-sensitive) tumors sediment as two distinctly separate 7-8 S and 12-13 S components (Figure 1B). Interestingly, [^{3}H]dexamethasone-receptor complexes present in tumors derived from the parental RPMI 3460 cell line also sediment both at 7-8 S and 12-13 S (data not shown). Although it is consistently present in the glucocorticoid-sensitive tumors (RPMI 3460 and clone 6), amounts of the 12-13S component vary between tumors. However, when tumor cytosols (either RPMI 3460, clone 5 or clone 6) are prepared and analyzed in buffer additionally containing 10mM sodium molybdate ($TDG_{25}M$), their [^{3}H]dexamethasone-receptor complexes are indistinguishable and sediment as narrow bands at 9-10 S (Figure 2). We have determined that this effect does not simply represent an irreversible loss of either 7-8 S or 12-13 S receptor activity (see later). Furthermore, these molybdate associated alterations in receptor size and heterogeneity can be induced by the addition of molybdate to cytosol originally prepared in its absence. Cytosol prepared from clone 5 tumors in TDG_{25} buffer was equilibrated, immediately prior to density gradient centrifugation, into $TDG_{25}M$ buffer. The buffer exchange was accomplished by rapid chromatography of the TDG_{25} cytosol through small (10-11ml) G-25 columns which had previously been equilibrated with $TDG_{25}M$ buffer. The cytosol was then centrifuged through sucrose

[1] The abbreviations for marker proteins are as follows: Cat (catalase), I (gamma globulin), H (hemoglobin), and C (cytochrome c).

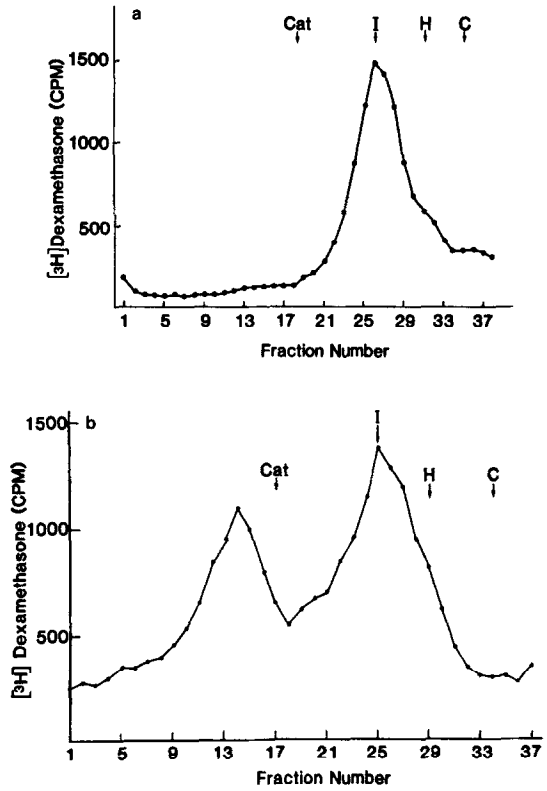

Figure 1: Sucrose density gradient analysis of glucocorticoid-receptor complexes prepared and maintained in TDG_{25} buffer. [^{3}H]dexamethasone labeled cytosol from glucocorticoid-resistant clone 5(a) and glucocorticoid-sensitive clone 6 (b) tumors.

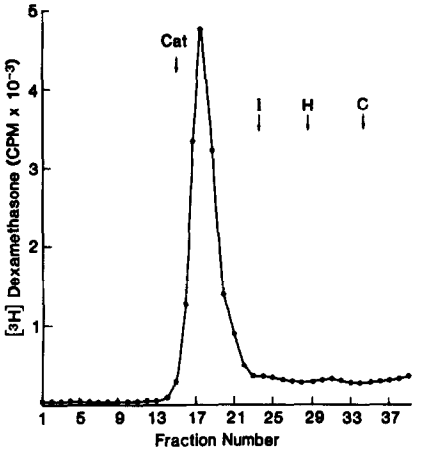

Figure 2: Sucrose density gradient analysis of glucocorticoid-receptor complexes prepared and maintained in $TDG_{25}M$ buffer. This profile represents [^{3}H]dexamethasone labeled cytosol from glucocorticoid-resistant clone 5 tumors. Identical data were obtained for RPMI 3460 and clone 6 tumors.

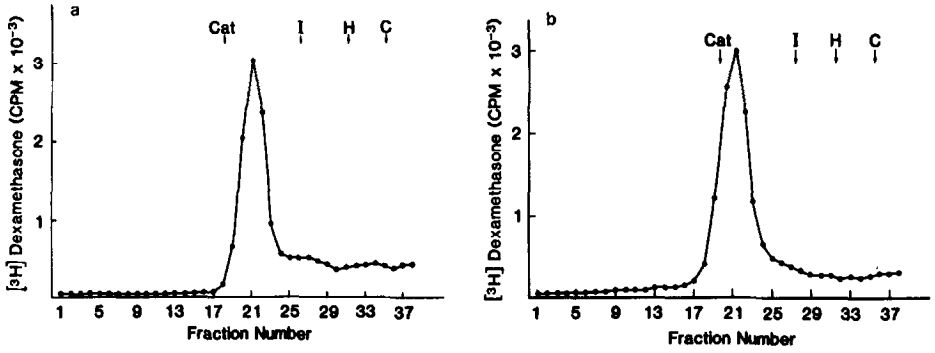

Figure 3: Alteration of sedimentation patterns by molybdate. [^{3}H]dexamethasone labeled cytosols were prepared in TDG_{25} buffer. Immediately prior to centrifugation, cytosols were equilibrated into $TDG_{25}M$ buffer (see Results). The profile shown in (a) is representative of glucocorticoid-resistant clone 5 tumor cytosols, while profile (b) represents typical data obtained for glucocorticoid-sensitive clone 6 and RPMI 3460 tumor cytosols.

density gradients also prepared with $TDG_{25}M$ buffer. As Figure 3A shows, the 7-8 S [^{3}H] dexamethasone-receptor complex characteristic of clone 5 cytosol in the absence of molybdate is missing and only a sharply sedimenting 9-10 S component is present. Similarly, when TDG_{25} cytosol from clone 6 tumors is equilibrated with $TDG_{25}M$ buffer and analyzed in an identical manner (see Figure 3B), both the 7-8 S and the 12-13 S receptor components are absent and only the sharply sedimenting 9-10 S component is present.

The molybdate induced alterations in receptor size and heterogeneity described above are reversible. Cytosols from RPMI 3460, clone 5 and clone 6 tumors were prepared in $TDG_{25}M$ buffer. Then, immediately prior to sucrose density gradient analysis, molybdate was removed using the G-25 buffer exchange technique outlined above. The 7-8 S receptor component from clone 5 tumor cytosol was regenerated from the molybdate-stabilized 9-10 S receptor component (Figure 4A). Similarly, as illustrated in Figure 4B for the case of RPMI 3460, both the 7-8 S and the 12-13 S receptor components present in RPMI 3460 and clone 6 tumors can be regenerated from the 9-10 S molybdate-stabilized component.

In contrast to results outlined above for the native cytosolic receptor, activated glucocorticoid-receptor complexes, isolated by DEAE-cellulose chromatography (18), are resistant to molybdate induced changes in size. Upon equilibration and analysis by

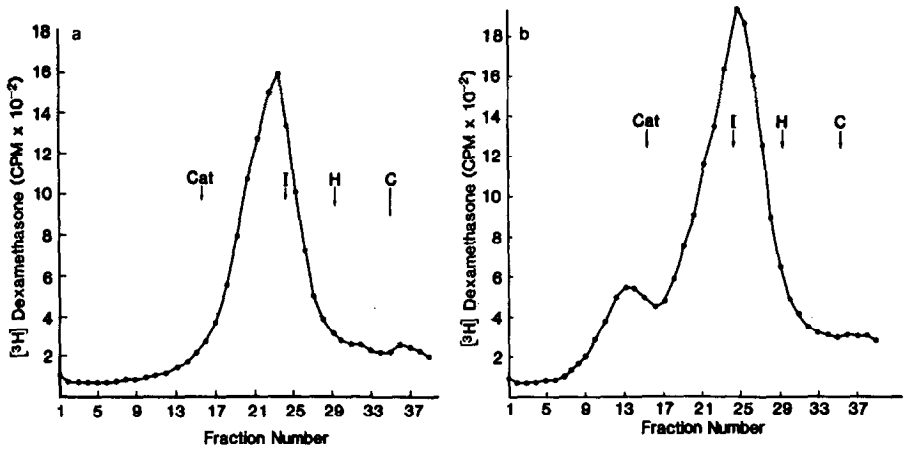

Figure 4: Reversibility of the molybdate effect. [^{3}H] dexamethasone labeled cytosols were prepared in TDG_{25} M buffer. Immediately prior to centrifugation, cytosols were equilibrated into TDG_{25} buffer (see Results). The profile shown in (a) is representative of glucocorticoid-resistant clone 5 tumor cytosols, while profile (b) represents typical data obtained for RPMI 3460 tumor cytosols.

sucrose density gradient centrifugation in either TDG_{25} or $TDG_{25}M$ buffer, the activated receptor isolated from either glucocorticoid-sensitive or -resistant tumors appears only as a 4-5 S component. These results are illustrated in Figure 5 for glucocorticoid-resistant clone 5 tumors. Data for activated receptor isolated from

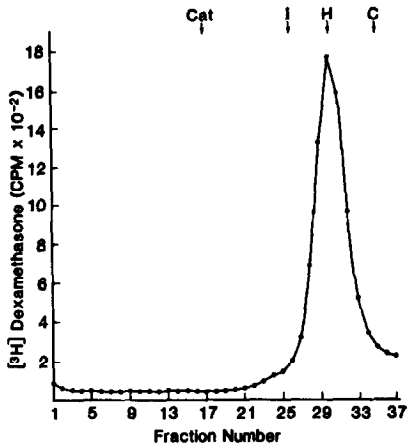

Figure 5: Analysis of activated glucocorticoid-receptor complexes on TDG_{25} sucrose density gradients. Activated glucocorticoid-receptor complexes were isolated from glucocorticoid-resistant clone 5 cytosol by DEAE-cellulose chromatography (see Methods).

RPMI 3460 and clone 6 tumors are identical (not shown). Note that passage of native receptors through Sephadex G-25 did not result in the appearance of activated receptor complexes (Figure 4).

Discussion

Several investigators have recently presented data showing that molybdate maintains glucocorticoid receptors as 9-10 S species during velocity sedimentation experiments (10,13). This type of molybdate stabilization has been observed for other classes of steroid hormone receptors as well (8,13). It has been suggested that the 9-10 S receptor complexes detected with molybdate resemble native holo-receptors (8) and that smaller forms (including 8S) are the products of degradation by endogenous enzymes (9,13). This designation of the 9-10 S component as the "native holo-receptor" has stemmed partly from the fact that larger, descrete forms of steroid-receptor complexes have not been clearly identified, particularly in the absence of molybdate. This is despite suggestive evidence to the contrary (8,20).

The inclusion of 25% glycerol in our sucrose density gradients has allowed us to demonstrate the simultaneous presence and provide resolution of two large, heterogeneous glucocorticoid-receptor complexes. Using these receptor complexes (from RPMI 3460 and clone 6 tumor cytosols) and also the 7-8 S receptor components alone (from clone 5 cytosol) as a basis for our investigation, we have shown that molybdate does not simply maintain, but actually induces the formation of a 9-10 S species from receptor components already present. Indeed, in our system, molybdate induction of the 9-10 S component involves both an increase (7-8 S$\rightarrow$ 9-10 S) and a decrease (12-13 S$\rightarrow$ 9-10 S) in the size of the glucocorticoid-receptor complex. We believe our demonstration of the induction and ultimate reversibility of molybdate effects on these receptor components argues against an enzymatic event controlling the interconvertibility of these various heterogeneous receptor forms.

The inability of molybdate to affect the sedimentation coefficient of salt-activated glucocorticoid-receptor complexes which have been isolated by DEAE-cellulose chromatography is interesting. It may reflect either a modification in receptor structure or the absence of a regulatory entity in these partially purified

fractions which may be required for molybdate stabilization of the receptor in its native conformation.

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