Characterization of a Unique Corticosterone-binding Protein in Candida albicans*

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This paper further characterizes a protein we have demonstrated in Candida albicans which has the ability to bind corticosterone and related steroid hormones. Fungal cells are disrupted and cytosol is incubated with [3H]corticosterone for 3 h at which time peak steady state binding is achieved. Bound hormone is separated from free using Sephadex G-50 minicolumns or dextran-coated charcoal. Binding was found to be a linear function of protein concentration. The bound hormone co-migrates with authentic corticosterone in thin layer chromatographic systems indicating no metabolism of the radioprobe. Scatchard analysis of the binding in the pseudohyphal form of C. albicans yielded values of 6.3 nM for the KD and a binding capacity of about 650 fmol/mg of cytosol protein; both determinations are comparable to our findings in the yeast form of this organism. A series of sterols were tested for their ability to displace [3H]corticosterone from the yeast binder, and the results show that the binder is remarkably selective and stereo specific. Physical-chemical studies show the binder to be degraded at high temperatures and that binding is destroyed by trypsin and sulfhydryl blockers. The protein sediments at 4 S on sucrose gradients and does not exhibit ionic dependent aggregation. The molecular weight is estimated to be ~34,000 daltons by gel chromatography. We hypothesize that this intracellular protein may represent a primitive form of either the mammalian glucocorticoid receptor or the plasma corticosteroid-binding globulin.

We have recently demonstrated the presence of a corticosterone-binding protein in the eukaryotic fungus Candida albicans and hypothesized that it may represent a steroid hormone receptor (1). It is not yet clear that this unicellular yeast actually utilizes hormones in the same sense that multicellular organisms do. However, molecules functioning in a fashion analogous to mammalian hormones have been demonstrated in several fungi. Gametogenesis in the yeast Saccharomyces cerevisiae is under the control of two mating-type specific peptides (2). In addition, Fusarium, a more complex fungus, has been shown to produce estrogenic-like molecules (3) which have the ability to bind to the mammalian estrogen receptor (4). Also, evidence has been adduced that the watermold Achlya bisexualis secretes steroids which regulate its reproductive processes in a manner similar to vertebrate hormones (5). However, to our knowledge a hormone receptor system has not previously been described in unicellular eukaryotes. In this paper we provide further biochemical characterization of the corticosterone-binding protein we previously identified in C. albicans (1). The protein exhibits many properties compatible with it being a receptor molecule; however, it is not surprising to find that there are major differences compared to mammalian glucocorticoid receptors. C. albicans also produces a lipid-extractable product which we hypothesize is the endogenous ligand for this binding protein and which will be described in a subsequent publication.1

EXPERIMENTAL PROCEDURES

Materials—Radioactive steroids were purchased from Amersham Corp. with the exception of R-5020 (Pronemestone) which was from New England Nuclear. 14C-labeled proteins, used for markers in sucrose gradients, were products of New England Nuclear. Most of the radiomimetic steroids were obtained from Steroids (Wilton, NH). Antheridial was a generous gift from Dr. Trevor McMorris (University of California, San Diego). Dexamethasone was a gift from Merck, and 1,25(Oh)D3 and 25(Oh)D3 were gifts from Hoffmann-LaRoche. Solvents used for extraction and chromatography were high performance liquid chromatography grade purchased from J. T. Baker Chemical Co. Sephadex (G-50 fine) and Sepharycl (S-200) were products of Pharmacia (Piscataway, NJ). Trasylol was obtained from Mobay Chemical Corp. (New York, NY). Other reagents were purchased from Sigma unless noted.

Yeast Cultures—The strain of C. albicans employed in these studies was derived from a clinical isolate kindly supplied by D. Schurman and identified serologically as type A in the laboratory of D. Stevens (both at Stanford University). Cultures were periodically examined using standard serological techniques to ensure that the cells used were unchanged. C. albicans were grown on nutrient agar (Difco) in 10-cm Petri dishes (Falcon, Oxnard, CA). Routinely, 12 dishes were streaked for heavy growth and cells were grown for 24 h at 37 °C before harvesting. Although most experiments employed the yeast form of the organism, the pseudohyphal form was also examined. Greater than 95% pseudohyphal cells were generated by growing the yeast in the presence of 5% newborn calf serum for 18 h at 37 °C. The serum was heated to 56 °C for 30 min to destroy corticosteroid-binding globulin (6) and was also charcoal stripped to eliminate endogenous steroids (7).

Steroid Binding Assays—C. albicans yeast forms were harvested by centrifugation at 1000 × g, and the packed cell pellet (~1.0 ml) was resuspended in 2 ml of homogenizing medium containing 250 μM sucrose, 10 mM Tris-HCl, 12 mM monothioglycerol, 1.5 mM EDTA, and 10 mM Na2MoO4, pH 7.8. An 0.5-ml aliquot of cells was added to 0.7 ml of 250–300 μM glass beads (Sigma) in 1.5-ml conical plastic centrifuge tubes. Cells were lysed by vigorous agitation on a Vortex mixer with repeated 15-s periods of cooling of the tubes in an ice slurry. Cortisol was then prepared by ultrafiltration at 204,000 × g for 30 min at 4 °C. In typical binding experiments, cytosol samples were incubated with [3H]corticosterone for 3 h at 0 °C. Preliminary experiments revealed that equilibrium was obtained under these conditions after 3 h at 0 °C.

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conditions. Nonspecific binding was assessed in all experiments by parallel incubations performed in the presence of a 250-fold molar excess of radioinert corticosterone. Bound steroid was separated from free steroid by chromatography of samples on 4 ml of Sephadex G-50 minicolumns as previously described (8). Specific binding was calculated by subtracting nonspecific binding from total binding. In some experiments, removal of free steroid was accomplished with a dextran-coated charcoal technique. Cytosol samples containing [3H]steroid were agitated for 15 s with a volume of 0.5% Norit A charcoal (Sigma) with 0.05% T-70 dextran (Pharmacia) equal to the incubation volume and centrifuged at 3000 × g to remove charcoal (9).

**Chromatographic Analysis of the Ligand to Evaluate Steroid Metabolism—**[3H]Corticosterone was examined for metabolic conversion after incubation both in the presence of cytosol and with intact C. albicans. Cytosol was prebound with 26 nM [3H]corticosterone for 3 h at 0 °C. Sephadex chromatography was performed as usual, and the protein peak was collected and extracted with methylene chloride. A second approach employed intact C. albicans cells incubated in serum-free Dulbecco’s medium (Grand Island Biological Co., Grand Island, NY) with 7 × 106 dpm/ml of [3H]corticosterone. After 48 h of incubation, yeast were removed by centrifugation, and the medium was extracted with methylene chloride. Both types of extract were dried under nitrogen and applied to 15-cm instant thin layer chromatography strips (Gelman Sciences, Inc., Ann Arbor, MI) and developed in hexane:chloroform:methanol, 80:20:2. Strips were then assessed for radioactivity with a radiochromatogram scanner (Packard Instrument Co.).

**Sucrose Gradient Analysis—**Cytosol was obtained by lysing cells in sucrose-free homogenizing medium in the absence (hypotonic) or presence of 0.3 M KCl (hypertonic). Cytosol samples (200 μl) were applied to 5–20% sucrose gradients which were prepared in either hypo- or hypertonic buffer. The gradients were centrifuged for 18 h at 337,000 × g to remove charcoal (9). Specific binding was determined with the dextran-coated charcoal technique.

**Molecular Weight Estimation—**Cytosol samples were applied to a calibrated Sephacyl S-200 column (90 × 1.6 cm) and eluted at a flow rate of 0.75 ml/min with a buffer consisting of 50 mM Tris and 1.5 mM EDTA, pH 7.6. Fractions (2 ml) were divided and incubated with 26 nM [3H]corticosterone ± 250-fold molar excess of radioinert corticosterone. Binding was determined with the dextran-coated charcoal technique.

**Enzymatic Studies—**Cytosol was prepared in modified homogenizing medium which contained no monothioglycerol or Na3MoO4. Trypsin (1:200, Difco), neuraminidase (Millipore Corp., Freehold, NJ), and N-ethylmaleimide (Sigma) were tested in this buffer; RNase and DNase I (Worthington) were tested in the presence of 6 mM MgCl2; phospholipase A2 (Sigma) was tested in the presence of 2.0 mM CaCl2. All fractions were incubated for 30 min at 37 °C, and then specific [3H]corticosterone binding was determined by the column chromatography method.

**Other Assays—**Protein concentration was measured by the Coomassie dye binding technique (10) employing a mixture of 80% human γ-globulin and 20% bovine serum albumin (Reheis Chemical, Phoenix, AZ) as the standard. All data are expressed as mean ± S.E. of specific binding.

**RESULTS**

**Existence of Specific Steroid-binding Sites in C. albicans—**Initial experiments to determine whether high affinity saturable steroid-binding sites were present in cytosol from C. albicans involved incubation of fungal cytosol with a variety of [3H]-ligands. As described previously, specific binding was found with [3H]corticosterone and [3H]progesterone (1). The following titrated probes failed to reveal specific binding: testosterone, dihydrotestosterone, estradiol, diethylstilbestrol, R-5020, dexamethasone, and triamcinolone acetonide, all tested at 26 nM and 1.25(0-2)D3 at 1.3 nM and 25(0-2)D3 at 26 nM. As noted, both [3H]corticosterone and [3H]progesterone exhibited specific binding. Cross-competition experiments revealed that both ligands were labeling the same binding site. As somewhat higher levels of specific binding were detected with [3H]corticosterone we have employed this steroid as our radioligand.

**Binding Assay Conditions—**Fig. 1 illustrates the time course of [3H]corticosterone binding to C. albicans cytosol at 0 °C. Binding is rapid, reaching maximal levels in 2 h, and remaining stable for several hours. Experiments at temperatures between 0 and 37 °C give similar results with similar levels of maximum binding detected although degradation occurs more rapidly at elevated temperatures. Most experiments were, therefore, performed at 0 °C after 3 h of incubation.

In further experiments to validate the binding assay we examined [3H]corticosterone binding as a function of cytosol protein concentration, and the results are shown in Fig. 2. Binding was found to be linearly related to protein concentration above 0.25 mg of protein/ml, and in subsequent studies we used this or a higher concentration of protein.

The binding reaction displayed quite a broad pH maximum, with little change in binding seen between pH 6.0 and 8.0 (data not shown). Buffer conditions were selected such that upon homogenization at 0 °C, cytosol pH was approximately 7.3–7.5.

**Proof that the Bound [3H]Corticosterone Is Unmetabolized—**It has been reported (11) that C. albicans possesses the ability to metabolize C-21 steroids. To ascertain whether the radioactive ligand bound in C. albicans cytosol was unmetabolized [3H]corticosterone, we performed a series of chromatographic analyses. The results of one such experiment are presented in Fig. 3. To assess the ability of the intact fungus...
to metabolize [3H]corticosterone, this steroid was incubated in a growing culture of C. albicans for 48 h. Medium was extracted with methylene chloride and chromatographed in a thin layer system in parallel with authentic [3H]corticosterone (Fig. 3a). As can be seen in Fig. 3b almost all the [3H]material extracted from the medium co-migrated with [3H]corticosterone. A small unresolved peak (~4% of the total, determined by peak area) migrates as a more polar entity. Based on the results of others (11) this material is probably a C-20 hydroxy derivative of corticosterone. In further studies [3H]corticosterone was incubated with cytosol from C. albicans and chromatographed over a Sephadex column. The protein peak after a typical binding experiment. Cytosol and growth medium were added to the cytosol. Samples were removed at the indicated times and assessed for specific [3H]corticosterone binding using the dextran-coated charcoal technique. The dissociation rate, k off, in this experiment is 0.043 min⁻¹. From three experiments k off = 0.042 ± 0.003 min⁻¹.

After establishing the appropriate conditions for the binding assay, we next examined various properties of the binding site.

Comparison of the Binding Protein in Pseudohyphal and Yeast Forms—We previously reported (1) that the cytosolic binder in the yeast form of C. albicans has an apparent equilibrium dissociation constant (Kd) of 7.2 ± 0.5 nM and a binding capacity of about 750 fmol/mg of protein. We were interested in examining the other morphologic form of C. albicans, the pseudohyphal form, to determine if there were changes in the binding protein to parallel the changes in morphology. Using suitable culture conditions, virtually all of the yeast forms can be converted to the pseudohyphal form. An isotherm and a Scatchard analysis of the [3H]corticosterone binding data for cytosol from the pseudohyphal form is shown in Fig. 4. Three such experiments yielded mean values for the Kd of 6.3 ± 1.6 nM and binding capacity of 653 ± 85 fmol/mg of protein, comparable to our results in the yeast form. These findings imply that the binding protein may serve a role in either form of C. albicans, and that at least within the limits of our assay, the protein is unchanged in affinity or binding capacity, even after a striking morphological change in the organism.

A role in either form of C. albicans.
Binding Kinetics—The binding of \([^{3}H]\)corticosterone to the fungal binder is rapidly reversible at 0 °C as shown in Fig. 5. The dissociation of the steroid-binder complex is apparently first order, as expected, and the rate constant calculated from the slope of the logarithmic plot is 0.043 min\(^{-1}\); the \(t_{1/2}\) for dissociation then being about 16 min. Given this rapid dissociation, our technique of separating bound hormone from free hormone underestimates the binding capacity of \(C.\) albicans.

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### Table I

| Competitor             | Molar ratio | % of control specific binding |
|------------------------|-------------|-------------------------------|
| Corticosterone         | 1           | 64 ± 3                        |
|                        | 10          | 15 ± 1                        |
| Cortisol               | 1           | 85 ± 10                       |
|                        | 10          | 55 ± 10                       |
| Prednisolone           | 10          | 83 ± 9                        |
|                        | 100         | 34 ± 2                        |
| 11α-Cortisol           | 10          | 90 ± 3                        |
|                        | 100         | 92 ± 2                        |
| Dexamethasone          | 100         | 114 ± 9                       |
|                        | 1000        | 96 ± 4                        |
| Triamcinolone acetonide| 10          | 100 ± 11                      |
|                        | 100         | 106 ± 3                       |
| Aldosterone            | 10          | 106 ± 7                       |
|                        | 100         | 94 ± 5                        |
| Progesterone           | 1           | 63 ± 3                        |
|                        | 10          | 13 ± 2                        |
| R-5020 (promegestone)  | 1           | 92 ± 5                        |
|                        | 10          | 72 ± 3                        |
| Testosterone           | 10          | 105 ± 13                      |
|                        | 100         | 85 ± 5                        |
| Estradiol              | 10          | 100 ± 5                       |
|                        | 100         | 103 ± 2                       |
| 1,25(OH)\(_{2}\)D\(_{3}\)| 10          | 93 ± 7                        |
|                        | 100         | 95 ± 8                        |
| Ergocalciferol         | 100         | 107 ± 18                      |
|                        | 1000        | 99 ± 11                       |
| Ergosterol             | 10          | 88 ± 5                        |
|                        | 100         | 97 ± 10                       |
| Cholesterol            | 10          | 83 ± 4                        |
|                        | 100         | 79 ± 5                        |
| Antheridiol            | 100         | 96 ± 3                        |
|                        | 1000        | 91 ± 8                        |

### Fig. 7.
Sucrose gradient analysis of the \([^{3}H]\)corticosterone binder in \(C.\) albicans. Sucrose gradients (6–20%) were prepared in hypotonic homogenizing medium (○) or in homogenizing medium containing 0.3 m KCl (□). The protein concentration of the hypotonic sample was 4.4 mg/ml while the hypertonic sample concentration was 5.9 mg/ml. After 200 µl aliquots of cytosol were layered on the gradients the tubes were centrifuged at 337,000×g for 18 h. Fractions (150 µl) were collected and incubated with 26 nM \([^{3}H]\)corticosterone ± 250-fold radiocorticosterone for 3 h at 0 °C to determine specific binding which is plotted in this figure. \(^{14}C\)-Markers, included in parallel gradients, were ovalbumin and bovine serum albumin with S values of 3.7 and 4.4, respectively.

### Fig. 8.
Molecular weight estimate of the \(C.\) albicans binder by Sephacyr chromatography. A cytosol sample (2 ml, 8.0 mg/ml) was applied to a Sephacyr column (90 × 1.6 cm) and eluted at a flow rate of 0.75 ml/min. Fractions of 2.0 ml were collected, split, and incubated with 26 nM \([^{3}H]\)corticosterone ± 250-fold excess radiocorticosterone to determine the specific binding values of which are plotted in this figure. The column was calibrated with globular proteins of known molecular weight including human γ-globulin (168K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), and RNase (13.7K). The molecular weight of the binder was estimated from a plot of \(K_m\) versus fraction eluted which was linear.
analysis of the steroid specificity of the binding in the yeast form is presented in Table I. Corticosterone and progesterone are potent and essentially equal in their ability to compete for $[^{3}H]$corticosterone-binding sites. Amongst glucocorticoids, cortisol and prednisolone are moderately good competitors, whereas 11α-cortisol (the inactive stereoisomer of cortisol), dexamethasone, and triamcinolon acetonide are without binding activity at the concentrations tested. Note that R-5020, the synthetic progestin with high affinity for mammalian progesterone receptors (12), is only about 2% as potent a competitor as progesterone. The other compounds assayed had minimal or no competitive activity at the concentrations tested. Ergosterol, the major sterol synthesized by this organism, is a very weak competitor. Mineralocorticoids, sex steroids, vitamin D metabolites, and ergocalciferol are all without activity. Antheridiol, a putative fungal hormone in Achyla bisexualis (5, 13) fails to compete at the concentrations tested. Taken together, these data indicate that the binder in C. albicans is remarkably steroid selective and stereospecific in nature.

**Binding Protein Stability**—We next focused on some of the properties of this binder using physical, chemical, and biochemical techniques. First, thermal stability and susceptibility to enzymatic digestion were explored. As shown in Fig. 6 the fungal binder is stable at 37 °C for 30 min but destroyed at 56 °C. After 30 min of incubation at 37 °C with 100 μg/ml of trypsin essentially all binding was abolished. Similar results followed exposure to 6 mM N-ethylmaleimide. DNase, RNase, phospholipase A, and neuraminidase had little or no effect on binding. These results suggest that the binder is a protein with free sulfhydryl groups required for binding.

**Sedimentation and Chromatographic Analysis**—Sucrose gradients have frequently been used in the study of mammalian steroid receptors, and the results of this technique used with the C. albicans protein are depicted in Fig. 7. The binder migrated at ~4 S in 5-20% gradients whether prepared in hyper- or hypotonic buffers. No disaggregation to smaller forms occurred in hypotonic gradients, a characteristic frequently noted with mammalian steroid receptors (14). In experiments not shown we did not see any changes in sedimentation coefficient when cytosol was prepared with protease inhibitors such as phenylmethylsulfonyl fluoride or trypsin.

To further assess the molecular size of this protein we employed chromatography over a Sephacryl S-200 exclusion gel column. These results are shown in Fig. 8. The broad based peak suggests heterogeneity of the binder. Since the binding assay was performed on the eluate fractions, this finding is not due to ligand dissociation during the chromatography procedure. Based on several experiments we estimate the molecular weight of the major binding protein peak to be ~43,000 daltons in hypotonic buffer. Taken with the sedimentation value of ~4 S, the fungal binding protein is somewhat asymmetrical (15).

**DISCUSSION**

In this report we have described an intracellular protein, found in C. albicans which binds corticosterone and certain other steroid hormones with high affinity, selectivity, and stereospecificity. We hypothesize that this protein represents a primitive form of either the mammalian glucocorticoid receptor or CBG. Even if this speculation does not prove to be correct, the protein is still of considerable interest.

Intense investigation into the evolution of steroid endocrine systems has occurred (for review see Ref. 16). Binding of mammalian hormones to a transport-protein-enzyme has been documented in Pseudomonas testosteroni (17) and seen after abiotic synthesis (18). In addition, a 5.1 S progesterone-binding protein has been reported in the bacterium Streptomyces hydrogenans (18). However, an intracellular steroid-binding protein with receptor-like properties has not previously been demonstrated in primitive eukaryotes. The fact that unicellular organisms may use sterols as message molecules appears to be well demonstrated in the fungus Achyla bisexualis where a C-29 sterol, antheridiol, seems to function via intercellular organisms may use sterols as message molecules appears to be well demonstrated in the fungus Achyla bisexualis where a C-29 sterol, antheridiol, seems to function via inter- 

- The CBG data is from several species taken from Westphal (20).
- The glucocorticoid receptor data are taken from several sources (14, 21, 22) which evaluate various organ sites including liver, thymus, and kidney.

| Property | C. albicans binder | CBG | Glucocorticoid receptor |
|----------|-------------------|-----|------------------------|
| Molar weight | 43,000 | 35,000 | 102,000 |
| Kd | ~4 | 3.4-4.1 | 4 |
| Hypotonic buffer | ~4 | 3.4-4.1 | 7-8 |
| $K_{d}$ | 7 | 1-7 | 3-30 |
| Stable at 37 °C/1 h | 0.04 | 0.027 | 0.003 |

The data presented here indicate that some unicellular fungi also possess intracellular steroid-binding proteins. We interpret this finding to imply that these simple organisms use such proteins as steroid/steroid receptors. However, we have examined several other simple organisms including Saccharomyces cerevisiae, Neurospora crassa, and Paracoccidioides brasiliensis and have not successfully demonstrated this corticosterone-binding site. Thus all fungi do not appear to possess a similar glucocorticoid binder.

The binding protein we have demonstrated in C. albicans exhibits some characteristics suggestive of mammalian receptors or specific plasma transport proteins; however, several properties of the fungal binder are clearly different. Table II compares the properties of the C. albicans binder with two well studied mammalian steroid-binding proteins, CBG, and the classical intracellular glucocorticoid receptor. Of the properties examined, the fungal binder resembles CBG somewhat more closely than the receptor although it is clearly not identical to either. Localization of the binder in the cytosol and the absence of secretion into the medium is suggestive of a receptor rather than a transport role for this binder. It would of course be interesting to establish whether the fungal binder has structural homology to mammalian receptors or CBG but

2 The abbreviation used is: CBG, corticosteroid-binding globulin.

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this approach to understanding the evolutionary role of this molecule is unfortunately not yet available to us. It is interesting to speculate on the possibility that the cytosol receptors and CBG may both have evolved from a primitive CBG-like molecule.

The affinity exhibited for corticosterone is similar in all three proteins. This glucocorticoid is the native ligand for both CBG and the receptor in rodents but is probably not the native ligand in C. albicans. We have described a lipid-extractable substance present in C. albicans and also released into the medium which has the ability to reversibly displace $[^{1}H]$corticosterone from the fungal binding site (1). We believe this material represents the natural ligand and it may prove to have a higher affinity than corticosterone at the fungal binding site. Until the active agent is available in pure form and radiolabeled the properties of the binding protein in the presence of the endogenous ligand cannot be evaluated.

The finding of a protein in a pathogenic fungus with the ability to interact with mammalian hormones raises the possibility that such organisms may respond to the host endocrine milieu via this binder. We are currently investigating this possibility.

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