Functional Characterization and Purification of an Intracellular Vitamin D-binding Protein in Vitamin D-resistant New World Primate Cells

AMINO ACID SEQUENCE HOMOLOGY WITH PROTEINS IN THE HSP-70 FAMILY *

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Most genera of New World primates exhibit resistance to vitamin D. These monkeys harbor high circulating concentrations of the prohormone 25-hydroxyvitamin D and the active vitamin D hormone 1,25-dihydroxyvitamin D. Previous work from this laboratory indicated that resistance is associated with the overexpression of a 60–65-kDa intracellular protein that binds vitamin D metabolites competitively. In the current studies 25-[3H]hydroxyvitamin D$_3$ (25-OHD$_3$) was used as a competitive ligand to investigate the ability of a number of small lipid molecules to interact with this intracellular vitamin D-binding protein (IDBP) in post-nuclear extracts of a prototypical lymphoblast cell line from the common marmoset, a vitamin D-resistant New World primate. Only those vitamin D metabolites with a hydroxyl moiety in the C-25 position were bound by IDBP. Disruption of the C-25 hydroxyl obviated binding, whereas more proximal alterations in the vitamin D side chain did not. Modifications in the A-ring of 25-hydroxylated vitamin D metabolites, most specifically hydroxylation of C-1, diminished but did not abolish ligand binding. Of more than two dozen other small lipid molecules examined, only the C-19 17-hydroxysteroids, 17β-estradiol and testosterone, and the C-21 progesterone were found to be capable of binding specifically to IDBP. Using a combination of physical and serial chromatographic techniques, we enriched IDBP 25-OHD$_3$ binding activity 17,588-fold in extracts of B95-8 cells. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of this purified fraction demonstrated a predominant 65-kDa molecular species with a pI 4.5. Seven different peptide fragments were isolated from the 65-kDa protein, each possessing sequence similarity to the hsp-70 family of proteins. Ligand binding analyses confirmed that human inducibly expressed hsp-70-bound 25-OH vitamin D$_3$ with approximately similar affinity (~10$^{-7}$ M) as did purified IDBP. In summary, these results suggest a novel action for the hsp-70 family of proteins as intracellular vitamin D- and gonadal steroid hormone-binding molecules.

With the notable exception of nocturnal primates in the genus Aotus (1), New World primates (NWP) exhibit vitamin D resistance (1, 2). This resistant state is characterized biochemically by high serum concentrations of the two major circulating vitamin D metabolites, 25-OHD and 1,25-(OH)$_2$D (1–3), and clinically by rickets in rapidly growing adolescent animals deprived of adequate sunlight exposure (4). Levels of 25-OHD may be up to 10-fold and 1,25-(OH)$_2$D up to 100-fold greater than those observed in Old World primates (OWP), including man. Unlike the majority of resistant states described for other steroid hormones and vitamin D in Homo sapiens (5, 6), resistance in NWP does not appear to be related to a mutation in the vitamin D receptor (VDR) protein (1, 7). Rather, the vitamin D-resistant state in NWP is associated with the apparent overexpression of an intracellular vitamin D-binding protein (IDBP) (1), which is distinct from members of the serum vitamin D-binding protein (DBP)/albumin families of proteins (8, 9). Unlike the cysteine-rich steroid/steroid-binding proteins in serum vitamin D-binding protein/albumin (9) and vitamin D/steroid receptor protein (10) families, which are confined principally to the extracellular domain and nucleus, respectively, IDBP is a relatively abundant, cysteine-poor protein concentrated in, but not confined to, the cytoplasmic compartment of cells (11). However, like these other steroid/steroid-binding proteins (8, 9), IDBP has been shown preferentially to bind 25-hydroxylated vitamin D metabolites (12). In the current report we have greatly expanded the vitamin D metabolite-IDBP binding studies to define more clearly which structural modifications of the vitamin D molecule are important for either enhancing or reducing ligand binding to IDBP extracted from NWP cells. We also document the IDBP binding potential of a much more extensive panel of molecules in an attempt to define additional classes of small, lipid-soluble signaling molecules which may interact with IDBP. Although we were previously able to enrich vitamin D metabolite binding activity in extracts of NWP cells (13), attempts to purify IDBP to homogeneity were unsuccessful. Here we report the purification and structural identification of the NWP IDBP as a member of the hsp-70 family of proteins and demonstrate the sterol/steroid binding properties of hsp-70-like proteins.

MATERIALS AND METHODS

Vitamin D Sterols, Steroids, and Other Compounds—25-[3H]Hydroxyvitamin D$_3$ (25-[3H]OHD$_3$; specific activity, 181 Ci/mmol) and

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Polytron on ice in five 15-s bursts. Nuclei, with associated nuclear steroid receptor proteins, were pelleted at 4,000 × g for 30 min at 4 °C. The nuclear pellet was discarded, and the supernatant was subjected to further centrifugation at 100,000 × g for 1 h at 4 °C. The supernatant was either aliquoted and stored at −70 °C for future study, used in competitive ligand binding analyses, or subjected to further purification.

**Chromatographic Enrichment of 25-[3H]OHD3 Specific Binding Activity in B95-8 Cell Extracts**—The 100,000 × g supernatant of the B95-8 cell extract was used in either competitive ligand binding analyses (see below) or as starting material for chromatographic enrichment of 25-[3H]OHD3 binding activity. Eleven different types of chromatographic matrices in open column, FPLC, and HPLC formats were evaluated to identify the optimal conditions for purification. The final sequence of column chromatography was: (i) ion exchange FPLC on BPS-DE cellulose (MetaChem) through an inclining linear NaCl gradient; (ii) hydrophobic interaction FPLC on phenyl-Sepharose (Pharmacia) through a declining NaCl stepwise gradient; and (iii) HPLC over HTP-hydroxyapatite (Bio-Rad) through a declining NaOH gradient. Protein was assayed according to the method of Bradford (14). The approximate molecular mass of 25-OHD3-binding moieties resolved with hydroxyapatite column chromatography was determined with size exclusion HPLC. Two pooled peaks containing specific 25-[3H]OHD3 binding activity were microconcentrated and desalted through a Centricon containing 30,000 Mw cut-off (Amicon, Beverly, MA), resuspended in mobile buffer of ETD, 0.5 mM NaCl (pH 7.3) and injected onto a Beckman TSK 4000PW size exclusion column (7.5 × 300 mm, Fullerton, CA) at a flow rate of 0.5 ml/min. 1.0-ml fractions were collected for analysis of specific hormone binding.

**Ligand Binding Analyses—**[3H]-Ligand binding was measured in crude B95-8 100,000 × g supernatants, in chromatographically enriched fractions, and in solutions of recombinant human inducible hsp-70 (0.05–50 μg/ml) with competitive protein binding assay (15). Crude material and post-anion exchange and post-hydrophobic interaction chromatography fractions were adjusted with NaCl-containing ETD buffer (pH 8.0) to achieve a final salt concentration of 0.5 mM NaCl for assay. Specific binding of 25-[3H]OHD3 to IDBP present in the traditional assay buffer of KETD (ETD containing 0.3 mM KCl (pH 7.4)) (16). Briefly, crude or post-FPLC fractions were incubated in ETD, 0.5 mM NaCl overnight at 4 °C with 4 nM 25-[3H]OHD3, in the presence or absence of 1–100 nM unlabeled competitive ligand. Protein-bound 25-[3H]OHD3 was separated from unbound steroid by incubation with dextran-coated charcoal. Specific binding 25-[3H]OHD3 was determined by subtracting the mean of duplicate determinations of binding in the presence of 100 nM radioinert competitive ligand (nonspecific binding) from the mean of individual fractions eluting from hydroxyapatite chromatography; the binding assay conditions were the same as those stated above except that specific binding in post-hydroxyapatite fractions was determined in ETD, 0.3 mM NaHPO4 buffer (pH 6.8).

**Gel Electrophoresis and Amino Acid Sequencing—**Post-hydroxyapatite fractions enriched for specific binding of 25-OHD3 were desalted, microconcentrated, and electrophoresed through an 11% discontinuous SDS-polyacrylamide electrophoresis gel as described by Laemmli (17) for 1 h at 200 volts. Visualization of resolved proteins was accomplished with silver staining (PhastGel, Pharmacia). The molecular weight of 25-OHD3-binding proteins was also estimated by specific in-gel binding of radiolabeled 25-[3H]OHD3. Microconcentrated, post-hydroxyapatite retentate was loaded onto adjacent lanes and subjected to SDS-polyacrylamide gel electrophoresis as described above. Following electrophoresis lanes of the gel were individually sliced, washed five times, each for 5 min, in ETD buffer at 4 °C, and then placed in a hybridization bag with 2.4 nM 25-[3H]OHD3 in ETD, 0.5 mM NaCl buffer in the presence or absence of 100 nM 25-OHD3. After overnight incubation at 4 °C on a rocker platform, the gels were washed several times in 25-OHD3 takedown buffer (18) containing 123 mM sodium barbital, 123 mM sodium acetate (pH 8.6), and 1 mM sodium azide without dextran or charcoal. The gel lanes were treated with Fluoro-Hance (RPI, Mt. Prospect, IL), dried under vacuum at 60 °C for 1 h, and cut horizontally into 5-mm slices. Each gel slice was placed into scintillation vials containing Cytoscint (ICN, Irvine, CA) and radioactivity measured.

To resolve further the protein species present in microconcentrated, fractionated post-hydroxyapatite retentates, aliquots of retentate were also subjected to two-dimensional electrophoresis as described by

### Table I

**Identity, 25-OH D3 binding index, and source of potential IDBP ligands**

| Compounds               | RBIa | Source                        |
|-------------------------|------|-------------------------------|
| DHEA and DHEAS, dehydroepiandrosterone and sulfate form, respectively | Sigma | Pharmacia                     |
| Pregnenolone            | Sigma| Pharmacia                     |
| Progesterone            | 19   | Sigma                         |
| Hydrocortisone          | 0    | Sigma                         |
| Cortisone               | 0    | Sigma                         |
| Corticosterone (B)      | 0    | Sigma                         |
| Deoxycorticosterone (DOC)| 0   | Sigma                         |
| Aldosterone             | 0    | Sigma                         |
| DHEA                    | 0    | Sigma                         |
| DHEAS                   | 0    | Sigma                         |
| Dexamethasone           | 0    | Sigma                         |
| Androstenedione         | 0    | Sigma                         |
| Androstanediol          | 0    | Sigma                         |
| Testosterone            | 30   | Sigma                         |
| 5-Dihydroytestosterone  | 35   | Sigma                         |
| 17b-Estradiol           | 33   | Sigma                         |
| Tamoxifen               | 0    | Sigma                         |
| RU486                   | 0    | Rousell-U.C.L.A.F.            |
| Cholesterol             | Sigma| Sigma                         |
| 25-Hydroxycholesterol   | Sigma| Sigma                         |
| Arachidonic acid        | 0    | Biomol                        |
| Prostaglandin E1        | 0    | Biomol                        |
| PGE2                    | 0    | Biomol                        |
| PGE2a                   | 0    | Biomol                        |
| All-trans-Retinoic acid | 0    | Sigma                         |
| 9-cis-Retinoic acid     | 0    | Sigma                         |
| Triiodothyronine        | 0    | Sigma                         |

a DHEA and DHEAS, dehydroepiandrosterone and sulfate form, respectively; PGF, proglandin.
RESULTS

Binding of Vitamin D Metabolites and Analogs

The ability of vitamin D₃ and various of its metabolites to displace 25-[³H]OHD₃ competitively in unfraccionated 100,000 × g supernatant extracts of B95-8 cells is shown in Fig. 1. When incubated with extract at a 100 nM concentration, the naturally occurring metabolites 25-OHD₃, 24,25-(OH)₂D₃, and 25,26-(OH)₂D₃ were roughly equivalent in their ability to inhibit 2 nM 25-[³H]OHD₃ binding competitively. The increased polarity, because of the presence of a C-1-α-hydroxy group, in 1,25-(OH)₂D₃ and 1,24,25-(OH)₃D₃ significantly decreased effective competitive binding; the C-1 position is the only naturally occurring site of metabolic hydroxylation in the A-ring of vitamin D₃ (26). Two synthetic compounds that possess a C-1-α-hydroxy group (1-OH₃D₃) or a pseudo-C-1-α-hydroxy group (dihydrotachysterol), but lack hydroxy substitutions in the molecular side chain, were also incapable of displacing 25-[³H]OHD₃. Vitamin D₃ that is not modified in the side chain and lacks a C-1 α-hydroxy was similarly ineffective as a competitive ligand. 25-[³H]OHD₃ displacement by serial dilution of those vitamin D₃ metabolites shown to be effective competitive inhibitors of 25-[³H]OHD₃ binding is shown in Fig. 1B. Assuming an 80% displacement of bound 25-[³H]OHD₃ as the maximal inhibition of binding, the ESD₅₀ for binding could be determined for only three of the five metabolites, 25-OHD₃, 25,26-(OH)₂D₃, and 24,25-(OH)₂D₃; 1,25-(OH)₂D₃ and 1,24,25-(OH)₃D₃ displaced 25-OHD₃ by only 20% at 10⁻⁷ M with no apparent displacement at lower concentrations. The ESD₅₀ for 25-OHD₃, 25,26-(OH)₂D₃, and 24,25-(OH)₂D₃ was 5 × 10⁻¹⁰, 5 × 10⁻¹⁰, and 5 × 10⁻⁸ M, respectively.

Vitamin D₃, a Δ⁵,7-diene steroid synthesized primarily in plants, differs structurally from vitamin D₃ in the side chain (Fig. 2A). In contrast to vitamin D₃ and its metabolites, vitamin D₃ and its metabolites possess a Δ⁵₉(C-22-C-23 double bond) and a C-24 methyl group. 25-OHD₃ and 1,25-(OH)₂D₃ were bound equivalently to 25-OHD₃ and 1,25-(OH)₂D₃ in unfractionated extracts (Fig. 2B). In comparison with modifications in the proximal portion of the side chain that did not alter sterol binding, structural changes in the terminal portion of the side chain did alter IDBP binding; for example, none of the C-1-hydroxylated, so-called “nonhypercalcemic” analogs MC903, EB1089, and KH1060 (Fig. 2A) was able to displace 25-[³H]OHD₃ binding in unfractionated B95-8 extracts (Fig. 2B).

Binding of Non-vitamin D Steroids and Bioactive Lipids

As depicted in Table I we also assessed the binding potential of a number of steroid precursor molecules, naturally occurring steroids, and two clinically useful steroid analogs, tamoxifen, and RU486. With the exception of progesterone, 17β-estradiol, and testosterone (12), none of these compounds was able to compete with 25-OHD₃ for binding in 100,000 × g supernatant extracts of B95-8 cells. This includes 25-hydroxycholesterol, a molecule that bears the C-3 and C-25-hydroxyl groups but lacks the Δ⁵,7-diene structure of the preferred seco-steroid. Interestingly, none of the six adrenal/gonadal steroid precursor

Fig. 1. Competitive displacement of 25-[³H]OHD₃ from NWP IDBP by vitamin D₃ sterols. Panel A shows the ability of C-25-, C-1-, and C-25-hydroxylated vitamin D₃ metabolites (100 nM) to compete with 4 nM 25-[³H]OHD₃ for specific binding to IDBP in B95-8 NWP cell extract. Metabolites (1α-OH₃D₃, analogs (dihydrotachysterol), and prohormones (vitamin D₃) lacking the C-25 hydroxyl group did not compete for ligand binding. Panel B shows the displacement of 4 nM 25-[³H]OHD₃ from IDBP through increasing concentrations of competitive 25-hydroxylated vitamin D₃ metabolites. All data are expressed as the percent maximal binding of 4 nM 25-[³H]OHD₃ in the absence of added competitor; each data point is the mean ± S.D. of at least three replicates.
of 25,26-dihydroxyvitamin D3 as displaceable ligand indicated that there was a specific 25-[3H]OHD3 binding activity through chromatography is depicted under “Chromatography” in Table II. Based on the relative stability and capacity of unfractionated 100,000 × g supernatant extract to bind 25-[3H]OHD3 at pH 8.0 (data not shown), elution of IDBP from a relatively “weak” anion exchange resin was achieved between 0.7 and 1.0 M NaCl. Fractions constituting the peak of sterol binding activity were pooled, applied to a phenyl-Sepharose FPLC column in a high ionic strength buffer, and eluted through a declining NaCl gradient in the absence of added detergent; phenyl-substituted resins provided the best recovery of specific binding activity. Although a substantial increase in specific 25-[3H]OHD3 binding activity was not obtained with hydrophobic interaction chromatography, it was critically important in separating the homodimer of glyceraldehyde-3-phosphate dehydrogenase from IDBP (27). Furthermore, chromatographic separation of glyceraldehyde-3-phosphate dehydrogenase and other proteins from IDBP on the basis of their hydrophobicity dramatically amplified the enrichment capability of hydroxyapatite FPLC for the protein(s) of interest.

There were two major peaks of specific 25-[3H]OHD3 binding activity in the eluent from hydroxyapatite chromatography (Fig. 3A), one eluting at ~50 mM (peak I) and a smaller one eluting at ~100 mM (peak II) Na2HPO4. Specific sterol binding was noted at no other position of the gradient. To assess the apparent molecular mass of these binding moieties an aliquot from the pooled fractions constituting peak I and peak II was subjected to gel filtration HPLC under non-denaturing conditions (Fig. 3B). Both peaks from hydroxyapatite chromatography exhibited specific 25-[3H]OHD3 binding activity in the range of 60–65 kDa. This was confirmed by in-gel labeling with 25-[3H]OHD3.

Considering that gonadal steroids were also bound in post-nuclear (12) as well as in 100,000 × g supernatant of the post-nuclear extract of B95-8 cells (Table I), we examined the possibility that one or both of the 25-OHD3 binding peaks from hydroxyapatite chromatography were also responsible for gonadal steroid binding observed in the unfractionated extracts of B95-8 cells. Aliquots, matched for protein concentration, from peak I and peak II were subjected to ligand binding analysis with tracer quantities of 25-[3H]OHD3 or 17β-[3H]estradiol in the presence and absence of 100 nM radio-inert 25-OHD3, and 17β-estradiol. Specific binding activity for 25-OHD3 exceeded that of 17β-estradiol by 21-fold (8.3 versus 0.2 nmol/mg of protein) and 17-fold (6.7 versus 0.2 nmol/mg of protein) in peak I and peak II, respectively. These data indicated that both peak I and peak II harbored 25-OHD3 and 17β-estradiol binding capacity, they were relatively enriched for specific 25-[3H]OHD3 binding activity compared with unpurified NWP cell extracts (see Table I).

GeL Electrophoresis and Amino Acid Sequence Analysis—Two-dimensional polyacrylamide gel electrophoresis of the 25-[3H]OHD3 binding moieties in peak I from hydroxyapatite chromatography (see Fig. 3A) identified at least three distinct proteins in the molecular mass range of interest (60–70 kDa).
The dominant, 65-kDa, Coomassie-stained spot displayed a pI ~ 4.5. Amino acid composition of the eluted protein(s) revealed the presence of very few cysteine residues (~4% the total residues), once again distinguishing this protein(s) from the cysteine-rich proteins in steroid/thyronine receptor superfamilies (10) and in the albumin family of proteins which includes the circulating vitamin D-binding protein (8, 9). Because the amino terminus of the most abundant protein in the isolate was blocked, we committed the remaining sample to in-gel proteolytic cleavage (21) prior to amino acid sequence analysis of the proteolytic products. Reverse-phase HPLC of the resultant peptides resulted in the separation and reproducible resolution of seven mass peaks belonging to the parent protein. The amino acid sequence of each was determined after Edman degradation of the peptide fragments. Sequence analyses identified major tryptic peptides ranging in length from 10 to 15 residues and possessing 89, 83, 75, 70, 60, 55, and 27% amino acid sequence identity to human inducibly expressed hsp-70 (28) (Fig. 4A). Two of the three tryptic peptides with most sequence homology to hsp-70 should be able to bind 25-[3H]OHD3 specifically. This was the case. Inducibly expressed hsp-70 exhibited specific 25-[3H]OHD3 binding activity (Fig. 4B). The concentration of radioinert 25-OHD3 required to achieve half-maximal displacement of 25-[3H]OHD3 from both post-hydroxypatitie-purified IDBP and from inducibly expressed hsp-70 was approximately the same, 5–10 nM.

**DISCUSSION**

The existence of steroid hormone-resistant states in monkeys from the New World was first described by Chrousos and his colleagues (29). They reported high circulating levels of adrenal 

(29–31) and gonadal (32) steroids in NWP from several different genera. Vitamin D resistance in NWP was first noted by Shink <et al.> (2) and confirmed by other investigators (33–35). NWP have adapted well to their resistance to gonadal and adrenal steroids (36). They do not suffer from adrenal insufficiency or infertility, presumably because of their ability to augment adrenal and gonadal steroid production from endogenous cholesterol substrates to meet physiological demands. Neither do they experience clinical vitamin D deficiency when residing in their normal habitat, the canopy of the equatorial rain forests of South and Central America.² It is only when these primates are imported and maintained in artificial environments at elevated latitudes that they develop the clinical syndrome of vitamin D deficiency; in fact, exposure of primates with clinical rickets or osteomalacia to an artificial source of ultraviolet radiation will dramatically increase circulating 25-OHD and 1,25-(OH)₂D concentrations and resolve their metabolic bone disease (4).

A number of theories have been put forward to explain the steroid- and vitamin D-resistant state in NWP. Some investigators have suggested that alteration in the structural character, quantity, and/or avidity of circulating steroid-binding proteins for ligand may contribute to the high circulating concentrations of hormone (37). However, we (33) and others (38) have not been able to discern a functional difference among the circulating steroid-binding proteins in OWP and NWP. Brandon <et al.> (39) suggested that steroid hormone resistance in NWP, as in humans, may result from changes (conserved

² A. Baker, personal communication.
mutations) in the receptor proteins for these ligands; these investigators have shown decreased receptor-ligand and receptor-DNA binding activity in NWP cells compared with OWP cells (32). However, specific function-altering mutations (i.e., coding for a premature stop codon) in the NWP receptor proteins, in either the ligand or DNA binding domains, have not been described (39). In fact, our experimental results with the VDR in NWP cells suggest that it is normal in all respects (1, 13). Furthermore, in evolutionary terms, the odds of independent, coincident, and conserved resistance-causing mutational events taking place in several of the steroid/sterol receptor genes in just the last 50 million years, since the occurrence of Old and New World continental drift (40), are very slim. More likely is the possibility that an alteration in expression of normally expressed gene products has been adopted to selective advantage in the suborder Platyrrhini (NWP).

Moore et al. (41) have suggested that glucocorticoid and mineralocorticoid resistance in NWP results in part from an alteration in the regulation of one such molecule, an enzyme, 11β-hydroxysteroid dehydrogenase, which catalyzes cortisol conversion to the biologically inactive metabolite cortisone. Decreased activity of this enzyme will promote accumulation of cortisol in the serum as is observed in the vitamin D2 molecules synthesized by plants, did not alter binding as long the C-25 hydroxyl moiety was present. This observation argues against the theory set forth by Marx and colleagues (43) to explain vitamin D resistance. These investigators postulated that there was a functional and structural standpoint.

We theorized that the C-1-hydroxylated metabolites of vitamin D₃, which bind most avidly to the VDR, would be most avidly bound by IDBP. This was not the case (Fig. 1). The C-25-hydroxylated metabolites of vitamin D₃, 25-OHD₃, 24,25-(OH)₂D₃, and 25,26-(OH)₂D₃, were the most tightly bound of the naturally occurring vitamin D₃ metabolites tested. In fact, hydroxyl substitution in the C-1 position of the A-ring of vitamin D₃ reduced, not increased, the affinity of the ligand for IDBP. We considered the possibility that IDBP, or a related protein, may be the long sought intracellular receptor protein for some of those vitamin analogs that have potent immunoinhibitory action in vitro and in vivo without causing hypercalcemia in the host (26). Three such synthetic compounds, which harbor structural alterations in the terminal aspects of the vitamin D side chain (Fig. 2) did not bind to IDBP. On the contrary, more proximal alteration of the vitamin D side chain, as occurs naturally in the vitamin D₃ molecules synthesized by plants, did not alter binding as long the C-25 hydroxyl moiety was present. This observation argues against the theory set forth by Marx and colleagues (43) to explain vitamin D resistance in NWP. These investigators postulated that there was preferential utilization of vitamin D₃ (cholecalciferol) metabolites over vitamin D₂ (ergocalciferol) metabolites in NWP cells.

The fact that 1,25-(OH)₂D₃ was apparently not the vitamin D ligand of choice for IDBP coupled with the finding that the structurally dissimilar gonadal steroid hormones 17β-estradiol, testosterone, and progesterone were also capable of specifically displacing 25-OHD₃ from binding protein(s) in NWP cell extracts (12), led us to speculate that (i) we had not yet identified the preferred ligand for IDBP and (ii) there was likely to be more than a single ligand-binding species in our...
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crude extract data. In an attempt to address the former we took a “candidate ligand” approach (Table I). Aside from confirmation of the fact that IDBP bound the three previously mentioned sex steroids, albeit with a relative binding index less than or equal to 25-hydroxylated vitamin D metabolites, there was no evidence for IDBP being able to bind any of the other 25 candidate ligands chosen for investigation. This list of candidate ligands included adrenal and gonadal steroid precursor molecules, metabolites and analogs, as well as other known bioactive lipid molecules. The relatively uninformative nature of this search for a potential candidate ligand led us to concentrate efforts on purification and structural identification of IDBP(s). Serial chromatographic purification of extracts of NWP cells showed IDBP to be a 65-kDa protein (Fig. 3). In contrast to previously evaluated crude whole cell extract (12), the material derived from serial chromatography in the current studies exhibited much higher specific 25-OH-D binding activity (Table II), a substantial decrement in specific 17α-estradiol binding, and complete loss of specific testosterone and progesterone binding (data not shown). Since we purified our extracts selectively on the basis of 25-OH-D binding, these results indicate that there are distinct binding proteins or IDBP isoforms that bind gonadal steroids in NWP cells.

We were surprised by the amino acid sequence data, which clearly demonstrated that the IDBP isolated here was a member of the hsp-70 family of proteins (Fig. 4); unlike some of the other molecular chaperones, including the immunophilins (44) and with the exception of sulfoglycolipids (45), hsp-70 and related proteins are not known to bind small lipophagic ligands specifically. Although they can be associated with multimeric cytoplasmic protein complexes that include steroid hormone receptors (46), members of the hsp-70 family of proteins have not been recognized previously for their potential to bind specific steroidal/steroid hormones. Moreover, the capacity of IDBP to bind 25-hydroxylated vitamin D sterols is apparently not confined to hsp-70-related proteins in NWP cells. A functional homolog of IDBP can be identified in OWP cells including those of human origin (11). Whether or not this homolog is the same homolog of IDBP can be identified in OWP cells including those of human origin (11). Whether or not this homolog is the same

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