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B700, an Albumin-Like Melanoma-Specific Antigen, is a Vitamin D Binding Protein

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B700, a murine melanoma-specific antigen, is a member of the serum albumin protein family. Other members include serum albumin and vitamin D binding protein. The primary structure and biochemical functions of B700, as well as its *in vivo* metabolic fate, are largely unknown. We compared murine albumin, vitamin D binding protein and B700 for their ability to specifically bind [^{3}H]-1,25-dihydroxy-vitamin D_3. Scatchard analysis revealed a single binding site for B700 with a K_a of 51 000 mol/l and a B_{max} of 4.51×10^{-7} mol/l. There was no significant difference in the K_a and B_{max} among the albuminoid proteins. However, differences in the binding sites could be distinguished by competition experiments where vitamin D_3, vitamin D_2 or 7-dehydrocholesterol competed for the specific binding of 1.25-dihydroxyvitamin D_3 to a greater extent by B700 than by vitamin D binding protein. The albumin binding site more closely resembles vitamin D binding protein than B700, but the data indicate that the binding function of the albuminoid proteins is conserved in B700.
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INTRODUCTION

B700, a 67 000 D glycoprotein, is one of the most specific and highly characterised melanoma antigens in mice [1–3]. Related molecules have been demonstrated on all cultured melanoma cells to date, including those of mice, hamsters, swine and humans [4]. This suggests that the B700-like proteins are "pan-melanoma" antigens. Evidence based on aminoacid composition, antibody crossreactivity, and N-terminal sequence characterises B700 as an albuminoid molecule [5, 6]. Other members of the serum albumin family of proteins include α-fetoprotein and vitamin D binding protein. While these criteria clearly place the B700 antigen in the serum albumin protein family, its biological/biochemical function(s) are unknown.

One way to elucidate the potential biochemical function of any protein is to determine the extent to which functional properties of related proteins have been conserved. Serum albumin possesses a variety of biochemical properties that range from ligand carrier to weak enzymatic activity [7]. We previously demonstrated that B700 can function as a prostaglandin-converting enzyme in the same manner as murine serum albumin (MSA) [8].

One function ascribed to albuminoid molecules is the well-known carrier function, in which many small molecules are transported in the circulation [7]. Of these, perhaps the most important specific binding function is the transport of vitamin D and its metabolites by vitamin D binding protein [7, 9]. Accordingly, the object of this study was to further characterise the potential biochemical functions of B700 by asking whether the vitamin D binding function has been conserved in B700. The most expedient way to study this was to tether B700, MSA and mouse vitamin D binding protein (MVDBP) to a solid phase and measure the binding of radioactive 1,25 dihydroxyvitamin D_3. We then compared the nature of the binding sites by monitoring the ability of other steroids to compete in the solid phase assay. It will thus be shown that the binding function for 1,25 dihydroxyvitamin D_3 has been largely conserved in B700.

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MATERIALS AND METHODS

Materials

MSA (fraction V), vitamin D_2 and 22-ketocholesterol were obtained from Sigma. Cholesterol was purchased from Applied Science Laboratories (State College, Philadelphia), 7-dehydrocholesterol from International Chemical (New York), and oestradiol from Supelco (Belleforte, Philadelphia). Mouse α-fetoprotein was from Calbiochem (La Jolla, California). Vitamin D_3 and 1,25-dihydroxyvitamin D_3 were kindly provided by Dr Uskokovic (Hoffman-La Roche, Nutley, New Jersey). MVDBP was provided by Dr Haddad (University of Pennsylvania). The protein was isolated from sera obtained from female B6D2F1 mice by actin affinity chromatography [10]. Dihydroxyvitamin D_3,1α,25-[26,27-^{3}H] (specific activity: 5.92 TBq/mmol), was obtained from DuPont–New England Nuclear (Boston, Massachusetts).

The B700 protein was isolated from solid B16 melanoma tumours [3, 11]. Briefly, C57B1/6N female mice obtained from the National Cancer Institute Frederick Cancer Research Facility (Frederick, Maryland) were inoculated subcutaneously with 10^6 cultured melanoma cells. Approximately 2 weeks later, the mice were killed by cervical dislocation and the solid tumours removed. The tumours were homogenised in a Waring blender and the B700 antigen was subsequently isolated by sequential purification using ammonium sulphate precipitation and preparative gel electrophoresis. The purified product was positively identified using rabbit anti-B700 antibody. Homogeneity was established by PAGE [12].

Vitamin D_3 binding assays

Solid phase radioactive capture assays were conducted using B700, serum albumin or vitamin D binding protein. The assays were conducted in multiwell dishes prepared as follows: equimolar amounts (100 ng B700, 100 ng MSA, or 73 ng MVDP) were bound per well to the surface of 96-well Immulon II dishes. The protein was added in a 0.1 mol/l sodium carbonate buffer. After the buffer evaporated to dryness, the wells were washed several times with phosphate-buffered saline. After washing, 50 μl 2 mg/ml soluble fish gelatin (Norland Products, New Brunswick, New Jersey) were added to each well to occupy the remaining unbound sites on the plastic. All subsequent binding assays were conducted using these treated plates.

Binding assays contained 100 ng protein and 12 650 dpm of [^{3}H]-1,25-dihydroxyvitamin D_3 (specific activity 5.92 TBq mmol/, DuPont–NEN) in Hank's buffered saline. The reaction mixture, containing either 0, 0.01, 0.05 or 1.5 μg unlabelled 1,25-dihydroxyvitamin D_3 in a total volume of 100 μl was incubated at 37°C for 2 h. The multiwell plates were washed with saline and the bound material was detached with 0.1 mol/l NaOH and quantitated in a liquid scintillation counter. Binding specificity was determined by measuring the concentration of the radiolabeled ligand bound under these conditions. Twelve-point Scatchard binding curves were performed by varying the concentration of the cold ligand over a concentration of 0.006 μg/well to 1.6 μg/well. Binding data from the Scatchard plots were analysed using the LIGAND computer program to determine the binding constants [13]. All binding assays reported in the results section were conducted in triplicate, and the data are reported as the mean (S.E.).

Competition experiments

1,25-dihydroxyvitamin D_3 assays were modified to examine the specificity of the binding sites on the albumin family proteins for other steroids. The normal assay procedure described above was modified by adding 2 nmol/well (approximately 1 μg) of non-radioactive 1,25-dihydroxyvitamin D_3, vitamin D_3, vitamin D_2, 7-dehydrocholesterol, cholesterol, oestradiol, 22-ketocholesterol or buffer alone. These assays were conducted for MSA, B700 or MVDP attached to the microtitre well plates. Each well also contained 5500 dpm of [^{3}H]-1,25-dihydroxyvitamin D_3, and the assays were incubated for 1 h at 37°C.

The effect of heat denaturation on the ability of the target proteins to bind 1,25-dihydroxyvitamin D_3 was determined by conducting assays in which 100 ng protein incubated at 100°C for 10 min or 100 ng of native protein was added to the standard reaction mixture in the wells. These assays were determined for MVDBP, MSA and B700.

RESULTS

The purpose of these studies was to determine whether the vitamin D binding function of albuminoids is conserved in B700. Toward this end three types of experiments were conducted: Scatchard analysis, competition by other steroids and heat denaturation. Operationally, the most convenient way to perform these studies was to use capture of tritiated 1,25-dihydroxyvitamin D [1,25$(OH)_2D_3$], the active form of vitamin D, by albuminoid proteins prebound to a solid phase. The solid phase method was chosen over a solution-based assay as a straightforward adaptation of our previous studies investigating the biochemical and immunological properties of B700 and MSA. These assays have been shown to be sensitive in distinguishing the two molecules immunochemically [1, 4, 6]. We

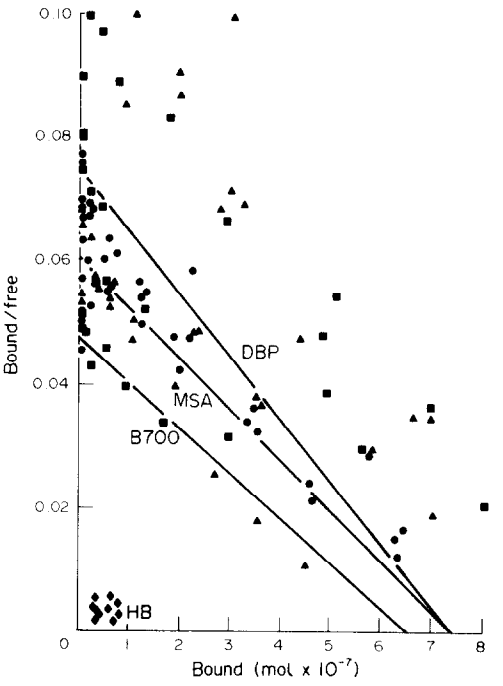

Fig. 1. Scatchard analyses of 1,25-dihydroxyvitamin D_3 binding by albuminoid proteins. Scatchard analyses (n = 3) were conducted for MVDBP (squares), B700 (circles) and MSA (triangles). Bovine haemoglobin (diamonds) was the negative control.

are alert to the fact that binding studies in solution are a far more accurate measure of the true K_a, especially since albumin-like molecules are "flexible" and change shape easily [7]. Nonetheless, experiments using the proteins bound to a solid phase are sufficient for comparative purposes.

Initial range-finding experiments (not shown) determined the optimal levels of ligand to be used and that these experiments could be conducted at 37°C for 2 h, as binding plateaued after 1 h under these conditions.

Scatchard analyses. Preliminary experiments also examined whether radiolabeled vitamin D_3 or 1,25-hydroxyvitamin D_3 was a more effective ligand for examining binding characteristics between the serum albumin family members. Low and inconsistent binding was observed when radiolabeled vitamin D_3 was used, while 1,25-dihydroxyvitamin D_3 provided reproducible and consistent results. Scatchard analyses were performed using 12–14 different concentrations of unlabeled material. The results of the Scatchard analyses are shown in Fig. 1 and the mean binding constants determined in three separate experiments are provided in Table 1. All of the serum albumin family proteins bound the [^{3}H]-1,25-dihydroxyvitamin D_3 with similar binding affinities (K_a) as determined from the slope of the Scatchard

Table 1. Binding parameters for 1,25-dihydroxyvitamin D_3 by serum albumin family members

Protein	K_a (S.E.)*	B_{max} (S.E)*
MVDBP	1.00×10^5 (4.20×10^4)	7.47×10^{-7} (1.34×10^{-7})
MSA	1.23×10^5 (4.30×10^4)	7.47×10^{-7} (2.73×10^{-7})
B700	1.39×10^5 (5.10×10^4)	6.57×10^{-7} (1.67×10^{-7})

*Mol/l.

Table 2. Percentage competition for 1,25-dihydroxyvitamin D_3 binding by other steroids

	Serum albumin family proteins		
Competitor (2 nmol/l)	MVDBP	MSA	B700
Buffer alone	0	0	0
1,25-dihydroxyvitamin D_3	44.6 (1.8)	27.2 (1.7)	50.1 (3.1)
Vitamin D_3	22.8 (1.5)	2.1 (0.1)	34.4 (7.0)
Vitamin D_2	5.1 (0.4)	0	46.5 (4.2)
7-dehydrocholesterol	31.3 (0.9)	31.8 (3.3)	41.8 (1.5)
Cholesterol	0	0	0
Oestradiol	0	0	0
22-ketocholesterol	0	0	0

Mean (S.E.), $n = 3$.

Table 3. Effect of heat denaturation on binding activity

Condition	Total binding	Non-specific binding	Specific binding
MVDBP			
Native	343 (12)	296 (19)	47
Boiled	450 (14)	260 (9)	190
Alone	496 (29)	239 (9)	257
B700			
Native	471 (25)	196 (7)	275
Boiled	459 (17)	194 (15)	265
Alone	536 (123)	208 (20)	328
MSA			
Native	515 (32)	261 (23)	254
Boiled	536 (31)	224 (9)	312
Alone	480 (24)	253 (17)	226

Disintegrations/min, $n = 3$.
Mean (S.E.).

plot. The S.E. for each of the values is rather large due to the larger values obtained in the third of the three Scatchard analyses. There were no significant differences between the K_a values when compared by a one-way analysis of variance with 17 degrees of freedom at the 0.5α level using the RS/Explore Data Program (BNN Software Products Corporation, Cambridge, Massachusetts). The F value from this analysis was 0.16, indicating that there was insufficient evidence to reject the null hypothesis that the samples came from populations with equal means ($P = 0.973$). No significant differences were detected between or within experiments. The similarity in the K_as among the various albuminoids for 1,25-dihydroxyvitamin D_3 was unexpected. Because the comparative results were reproducible within experiments, we decided not to conduct additional Scatchard experiments that might reduce the S.E. These data are sufficient to indicate that the binding domain is conserved.

The Scatchard binding data were analysed by the LIGAND programme to determine if a one site or a two site model would provide the best fit. A one binding site model provided the best fit for each of the serum albumin family members in each of the three experiments. The B_{max}, the maximum concentration of 1, $25(OH)_2D_3$ that can be bound per mol protein, was determined for each of the proteins from the Scatchard analyses and is provided in Table 1. The B_{max} values varied less than the K_a values. The S.E.s for the B_{max} values were about 20% or less than the mean value (Table 1). A one-way analysis of variance was performed on the B_{max} data using 17 degrees of freedom, with an F value of 0.49. There was insufficient evidence to reject the null hypothesis that the samples come from populations with equal means ($P = 0.78$), again indicating conservation of domain.

Specificity of vitamin D binding site

The specificity characteristics of the vitamin D-binding site of the albumin family members was compared using competition protocols. Non-radioactive vitamin D compounds and other steroids were mixed in solution (2 nmol/well) with 5500 dpm of [^{3}H]-1,25-dihydroxyvitamin D_3. This concentration was approximately 1 μg of unlabelled steroid per assay well. The results varied with the protein examined (Table 2). MVDBP exhibited the greatest specificity for 1,25-dihydroxyvitamin D_3 binding. The vitamin D_3 precursor 7-dehydrocholesterol inhibited radiolabelled 1,25-dihydroxyvitamin D_3 binding by MVDBP more effectively than an equivalent molar concentration of either vitamin D_3 or vitamin D_2. Vitamin D_3 was slightly more effective than vitamin D_2. Cholesterol, 22-ketocholesterol and oestradiol did not compete for the binding site, but increased binding of the radiolabel slightly. The specificity of binding by MSA was less than that of MVDBP. 7-dehydrocholesterol and 1,25-dihydroxyvitamin D_3 competed equally well for the binding site on MSA. Vitamin D_3, vitamin D_2, cholesterol, 22-ketocholesterol and oestradiol were not inhibitory at this concentration. The binding of radiolabelled 1,25-dihydroxyvitamin D_3 by B700 is less specific than that observed for MSA and MVDBP. This is illustrated by the fact that binding of the radiolabel can be inhibited by 1,25-dihydroxyvitamin D_3, vitamin D_3, vitamin D_2 and 7-dehydroxycholesterol. Cholesterol and 22-ketocholesterol were not inhibitory at the concentration tested, and appeared to slightly increase the specific binding of the radiolabel.

Heat denaturation of binding site

The importance of the tertiary structure of these proteins for ligand binding was examined by boiling the proteins to denature them. 100 ng of MSA, MVDBP or B700 was heated in boiling water for 10 min and added to the binding assays (100 ng/well), as described in Methods. Adding native MVDBP effectively competed with the MVDBP attached to the microtitre well for binding the radiolabelled 1,25-dihydroxyvitamin D_3 (Table 3). Boiling the MVDBP eliminated its ability to bind [^{3}H]-1,25-dihydroxyvitamin D_3. However, denaturation had no effect on the ability of either B700 or MSA to bind the radiolabelled vitamin D_3. The apparent increased binding observed in the assays to which boiled MSA was added was not statistically significant by t test. Although there appears to be a difference between the B700 boiled group and the B700 alone group, this difference is not significant due to the higher S.E. of the B700 alone.

DISCUSSION

The demonstration that the B700 murine melanoma antigen retains the vitamin D binding function of other albuminoids is significant for two reasons. Firstly, the data on the functional experiments further support the previous assignment of B700 to the albumin protein family based on structural criteria [5, 8, 14]. Secondly, the data provide information on a possible function or secondary function for B700 in binding,

transporting, and/or sequestering $1,25(OH)_2D_3$. Indeed, $1,25(OH)_2D_3$ therapy has been reported for melanoma tumours [15, 16]. Although traditional $1,25(OH)_2D_3$ receptors are present in B16 cells [17], an additional B700-mediated mechanism of entry into the cell can now be envisioned.

Our study shows that B700 binds 1,25-dihydroxyvitamin D_3 with a binding affinity similar to that of MVDBP and other serum albumin family members (Table 1). The binding constant, 10^5 mol/l, is lower than might be expected, and it is interesting that the serum albumin family members could not be distinguished by differences in their binding constants for 1,25-dihydroxyvitamin D_3 in this solid phase capture assay.

The binding constant for human vitamin D-binding protein in serum is 7×10^8 mol/l and for albumin 6×10^5 mol/l [18]. We attribute the differences in binding constants for vitamin D binding protein found in this study to the fact that a solid phase assay was used in our experiments. The observation (Table 3) that the native vitamin D binding protein in solution competes far more effectively for tritiated $1,25(OH)_2D_3$ than B700 or MSA did supports this view. Additionally, Scatchard analyses of 25-hydroxy vitamin D_3 binding and other vitamin D binding assays are influenced by several factors including buffer composition, incubation, temperature and vitamin D solubility. Difficulty in reproducing 25-hydroxyvitamin D Scatchard analyses has been reported [19].

The binding affinities of 1,25-dihydroxyvitamin D_3 by the various serum albumin family members were not different enough to delineate possible structural differences. However, differences in the specificity of these binding sites for other steroids were apparent (Table 2). Of the steroids tested, only 7-dehydrocholesterol, a precursor of vitamin D_3, competed with 1,25-dihydroxyvitamin D_3 for binding by MVDBP. Neither vitamin D_2 nor vitamin D_3 competed at the concentration tested. Binding by MSA showed close agreement with MVDBP in binding specificity because 7-dehydrocholesterol was the only steroid of those tested that competed for the binding site. The binding site on B700 was less specific than MSA or MVDBP. Vitamins D_2 and D_3 and 7-dehydrocholesterol competed for the 1,25-dihydroxyvitamin D_3 binding site. This aspect is interesting because cholesterol and 7-dehydrocholesterol have most of the same basic features, yet cholesterol did not compete for the binding site on the B700 protein. The competition for binding to MVDBP and MSA (Table 2) by 7-dehydrocholesterol in the absence of appreciable competition by cholesterol and vitamin D_2 was an unexpected finding. Apart from the obvious explanation of different cloud configurations, one would not have predicted these results from structural considerations. What is more significant, however, is the competition on the part of vitamin D_2 for binding to B700 because this suggests a divergence from the more common albuminoids.

Boiling inactivated the specific binding of MVDBP but not MSA and B700 (Table 3). The inactivation of MVDBP specific binding indicates that the tertiary structure is important in the specificity of the binding site on MVDBP. Interestingly, because one would predict similar binding sites on these proteins in view of their similar kinetic constants, the inability of boiling to inactivate binding by MSA or B700 raises questions regarding the importance of the tertiary configuration of their binding sites. The possibility that the binding site on serum albumin might be protected by the cystine bridges can be discounted because vitamin D binding protein is numerically and spatially similar to MSA [20–22]. The locations of the three cystines in B700 are not known. The B700 binding site is less specific for 1,25-dihydroxyvitamin D_3 binding (Table 2) and minor changes in the conformation may not affect this binding site to the extent observed for MVDBP. An additional consideration when evaluating the data of Table 3 is the well-known propensity of albuminoid molecules to dimerise [23]. In this context, one cannot account for the changes in the capture of radioactivity caused by the aggregation of heat-denatured protein in solution with bound molecules. This may account for the variation shown in Table 3 in which the native MVDBP but not native B700 or MSA is apparently able to compete for binding of the radioligand. However, this difference is not surprising in the light of previous studies in other species which have demonstrated the higher binding affinity of MVDBP compared to serum albumin [7, 9, 20].

Taken together, these data clearly demonstrate that the binding function of albuminoid molecules is conserved in B700. While the observed binding constant for vitamin D-binding protein was markedly lower than that observed in other studies, the data are sufficient to prove the conservation of function. Not surprisingly, B700 binding characteristics (specificity and heat-inactivation studies) more closely resemble those of MSA than of vitamin D binding protein. While the participation of B700 in antigen-mediated entry of $1,25(OH)_2D_3$ remains to be studied, demonstration of albumin-like function for B700 is equally important because these molecules have recently been postulated to be mediators of intercellular recognition [14].

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Feature Articles

EORTC New Drug Development Office Coordinating and Monitoring Programme for Phase I and II Trials with New Anticancer Agents

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INTRODUCTION

THE NEW DRUG DEVELOPMENT OFFICE (NDDO) is part of the Research Branch of the EORTC, being the executive office of the New Drug Development Coordinating Committee (NDDCC). The NDDO is directly involved in the coordination of all preclinical and early clinical steps in the development of new anticancer agents, i.e. the acquisition of new candidate compounds, the performance of *in vitro* and *in vivo* drug screening, the preparation of a suitable drug formulation for clinical use, the production of drug in enough quantities for toxicology and early clinical studies, the generation of animal toxicology data to allow a safe starting dose in humans, and the planning, data handling and monitoring of phase I and early phase II trials (Table 1) [1, 2].

Considering the importance of study monitoring for the achievement of high-quality standards of clinical trials and for the acceptancy of the data by the scientific community and regulatory authorities, it is very important that the procedures adopted by different monitoring organisations are frequently re-evaluated and discussed on the basis of the ethical and scientific values. The current operational procedures applied by the NDDO for the monitoring of phase I and II trials strictly follow the recommendations of good clinical practice (GCP) for trials on medicinal products, as defined by the Commission of the European Communities [3]. These guidelines are summarised in this paper.

PHASE I TRIALS: STUDY END-POINTS AND METHODOLOGY

When a new compound has completed preclinical evaluation, the first methodological step towards its clinical development is the phase I trial. In this type of study, patients with progressive malignancies no longer amenable to any form of available anticancer therapy are invited to participate in an experimental trial with a new anticancer agent. In phase I trials, the main

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