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Application of an allosteric model to describe the interactions among retinol binding protein 4, transthyretin, and small molecule retinol binding protein 4 ligands

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ABSTRACT

Retinol binding protein 4 (RBP4) is a serum protein that serves as the major transport protein for retinol (vitamin A). Recent reports suggest that elevated levels of RBP4 are associated with insulin resistance and that insulin sensitivity may be improved by reducing serum RBP4 levels. This can be accomplished by administration of small molecules, such as fenretinide, that compete with retinol for binding to RBP4 and disrupt the protein–protein interaction between RBP4 and transthyretin (TTR), another serum protein that protects RBP4 from renal clearance. We developed a fluorescence resonance energy transfer (FRET) assay that measures the interaction between RBP4 and TTR and can be used to determine the binding affinities of RBP4 ligands. We present an allosteric model that describes the pharmacology of interaction among RBP4, TTR, retinol, and fenretinide, and we show data that support the model. We show that retinol increases the affinity of RBP4 for TTR by a factor of 4 and determine the affinity constants of fenretinide and retinyl acetate. The assay may be useful for characterizing small molecule ligands that bind to RBP4 and disrupt its interaction with TTR. In addition, such a model could be used to describe other protein–protein interactions that are modulated by small molecules.

Retinol binding protein 4 (RBP4)[1] is a serum protein that serves as the major transport protein for retinol (vitamin A) [1]. The majority of RBP4 is produced in the liver, the site of storage and clearance of dietary retinoids, and is secreted into the plasma as an RBP4–retinol complex that delivers retinol to extrahepatic tissues. RBP4 is also expressed in adipocytes [2], and recent evidence suggests that it may function as an adipokine involved in metabolic homeostasis. For example, elevated levels of RBP4 are associated with insulin resistance in rodents and humans, and loss of RBP4, through either genetic or chemical means, results in improved insulin sensitivity in rodents [3]. Consequently, reducing RBP4 levels may be therapeutically beneficial for patients with type 2 diabetes.

RBP4 is a single polypeptide protein with a molecular weight of 21 kDa. In the serum, however, it is normally bound to another protein called transthyretin (TTR) in a 1:1 molar ratio. The resulting 76-kDa complex is large enough to protect RBP4 from being filtered through the kidneys and into the urine. ApoRBP4 (i.e., RBP4 devoid of retinol) has reduced affinity for TTR [4]; thus, after delivery of retinol to target tissues, the protective effect of TTR is lost and the apoprotein is cleared from the circulation.

Crystal structures have been solved for both apoRBP4 and retinol-bound RBP4, and they reveal that RBP4 contains a single binding site for retinol [5,6]. The ligand binding pocket is formed by eight antiparallel β-sheets that open into the solvent, with three structurally distinct loops that connect the ends of the sheets defining the entrance to the pocket. The molecule is oriented with the cyclohexene ring buried within the internal cavity and the hydroxyl group pointing toward the exterior of the protein. The structure of the retinol–RBP4–TTR complex has also been solved [7], revealing multiple contacts between the entrance loops and TTR as wells as a hydrogen bond between the hydroxyl group on retinol and the carbonyl of G83 of TTR.

Fenretinide [*N*-(4-hydroxyphenyl)retinamide] is a synthetic retinoid that was first synthesized during the late 1960s [8]. It is structurally similar to retinol but contains a phenylamide in place of the hydroxyl group. The crystal structure of RBP4 bound to fenretinide has also been solved, and it shows a binding mode very similar to that of retinol, with the cyclohexene ring buried within the internal cavity and the phenylamide pointing to the exterior of the protein [9]. Because the phenylamide occupies more space than the hydroxyl group of retinol, it causes positional changes in the entrance loops of RBP4 and, in addition, creates a steric hindrance in the region of the TTR–RBP4 binding interface. As a result, RBP4 that is bound to fenretinide cannot interact with

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[1] *Abbreviations used:* RBP4, retinol binding protein 4; TTR, transthyretin; FRET, fluorescence resonance energy transfer; APC, allophycocyanin; HTS, high-throughput screening; DTT, dithiothreitol; Ni–IMAC, nickel-immobilized metal affinity chromatography; SPA, scintillation proximity assay; PS, polystyrene; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; TR–FRET, time-resolved FRET.

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TTR. A consequence of this is that administration of fenretinide to animals and patients results in a rapid decrease of serum retinol and RBP4 levels due to renal clearance of RBP4 [10,11].

Fluorescence resonance energy transfer (FRET) assays have been used to measure protein–protein interactions in a variety of experimental systems. The methodology is dependent on the use of donor and acceptor molecules (typically both fluorophores) with overlapping emission and absorption spectra. When brought into close proximity, such as when each is coupled to one partner of an interacting pair of proteins, excitation of the donor (e.g., europium cryptate) results in energy transfer to the acceptor (e.g., allophycocyanin [APC]), which then emits light at a wavelength distinct from that of the donor. Consequently, agents that increase the protein–protein interaction will increase the FRET and those that disrupt the interaction will decrease the FRET.

The affinities of retinol and several other retinoids for RBP4 have been determined by fluorometric titrations [11–13]. In the current report, we describe an allosteric model to explain and characterize interactions involving RBP4, RBP4 ligands, and TTR. The model accommodates molecules that enhance (e.g., retinol) and disrupt (e.g., fenretinide) the RBP4–TTR interaction. The model was validated through the use of a FRET assay, which allowed determination of the affinity constants and cooperativity. We apply the model to aid in the design of molecules with improved potency over a disruptor that was identified from high-throughput screening (HTS).

Materials and methods

Chemicals

Retinol, fenretinide, retinyl acetate, and all other chemicals were purchased from Sigma (see Fig. 1 for structure of compounds used).

Recombinant human RBP4

Human TTR was purchased from Sigma. Human RBP4 containing an N-terminal hexa-histidine tag was expressed as an insoluble protein in *Escherichia coli*. The protein was recovered in inclusion body fractions and was reduced with 25 mM Tris–HCl (pH 9.0), 7.5 M guanidine HCl, and 10 mM dithiothreitol (DTT). The solubilized RBP4 protein was then refolded by diluting the solution 1:25 in 25 mM Tris–HCl (pH 9.0), 3 mM cysteine, 0.3 mM cystine, 0.7 M guanidine HCl, and 100 μM retinol. The refolding was performed for 48 h at 4 °C in the dark. After refolding, insoluble material was removed by centrifugation at 58,400*g* for 20 min. Refolded protein was purified to homogeneity by nickel-immobilized metal affinity chromatography (Ni–IMAC) followed by anion exchange chromatography on a Source Q column. The overall refolding and purification yield was greater than 1 mg per gram of wet cell paste (~60 mg/L of *E. coli* culture). The purified RBP4 (holo-RBP4) had three pairs of disulfide bonds and contained retinol. ApoRBP4 was produced by extracting the bound retinol from the purified holo-RBP4 with ice-cold ethyl ether. The extracted RBP4 was then purified by size exclusion chromatography on a Superdex 75 column to remove the remaining ether. Biotinylation of RBP4 and TTR was performed using the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce) according to the manufacturer's instructions.

Scintillation proximity radioligand binding assay

Saturation binding experiments were performed in white 384-well plates (OptiPlate, PerkinElmer) in a final assay volume of 50 μl per well. Each well contained 10 nM recombinant human biotinylated apoRBP4 and 0.075 mg of streptavidin–scintillation proximity assay (SPA)–polystyrene (PS) beads (GE Life Sciences) in SPA buffer (20 mM $NaPO_4$ [pH 7.5], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.02% Tween 20, and 0.01% bovine serum albumin [BSA]). [^{3}H]Retinol (45.5 Ci/mmol, PerkinElmer) was used as the radioligand, and nonspecific binding was determined in the presence of 30 μM unlabeled retinol. Reactions were incubated for 4 h at room temperature and measured with a ViewLux 1430 Ultra High Throughput Screening Microplate Imager (PerkinElmer).

FRET assay

The FRET assay was performed in black 384-well plates (OptiPlate) in a final assay volume of 30 μl per well. Reactions contained 5 nM recombinant human 6× His–apoRBP4, 100 nM biotinylated human TTR, 1 nM europium-labeled anti-His antibody, and

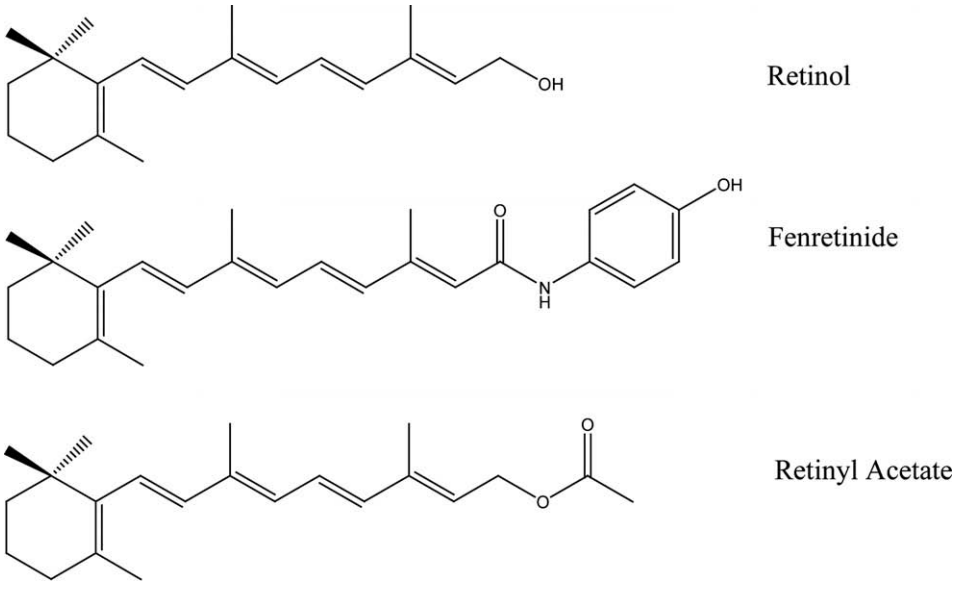

Fig. 1. Structure of compounds used in this study.