Published in final edited form as:

FEBS Lett. 2016 August ; 590(16): 2527–2535. doi:10.1002/1873-3468.12303.

Direct protein-protein interactions and substrate channelling between cellular retinoic acid binding proteins and CYP26B1

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Abstract

Cellular retinoic acid binding proteins (CRABPs) bind *all-trans*-retinoic acid (*at*RA) tightly. This study aimed to determine whether *at*RA is channeled directly to cytochrome P450 (CYP) CYP26B1 by CRABPs, and whether CRABPs interact directly with CYP26B1. *at*RA bound to CRABPs (holo-CRABP) was efficiently metabolized by CYP26B1. Isotope dilution experiments showed that delivery of *at*RA to CYP26B1 in solution was similar with or without CRABP. Holo-CRABPs had higher affinity for CYP26B1 than free *at*RA, but both apo-CRABPs inhibited the formation of 4-OH-RA by CYP26B1. Similar protein-protein interactions between soluble binding proteins and CYPs may be important for other lipophilic CYP substrates.

Keywords

Cytochrome P450; substrate channeling; retinoic acid; binding proteins

Introduction

All-trans-retinoic acid (*at*RA), is the main active metabolite of vitamin A and is responsible for many of the biological effects of vitamin A [1–3]. The cellular activities of *at*RA are believed to be mediated by a network of proteins including the enzymes synthesizing *at*RA, the CYP26 enzymes that are the major *at*RA clearing enzymes, retinoic acid receptors (RARs) and cellular retinoic acid binding proteins (CRABPs) [1–4]. Inside cells *at*RA is predominantly bound to its cellular binding proteins CRABP-I and CRABP-II [5,6] while in plasma, *at*RA is believed to be bound to albumin. *at*RA acts as a transcriptional activator by binding to specific transcription factors such as RARs, and the transcriptional activity of *at*RA has been shown to be increased by CRABP-II expression [7–9]. The effect of CRABP-II on RAR activation is likely due to the fact that *at*RA is channeled by CRABP-II but not CRABP-I to RARα, thereby facilitating transcriptional activity [8]. It has also been shown that in response to *at*RA binding, CRABP-II localizes to the nucleus whereas CRABP-I does not [7]. However, in the absence of *at*RA, CRABP-II co-localized with the endoplasmic

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reticulum (ER) [10]. In contrast, increased expression of CRABP-I was shown to decrease the differentiation-specific gene expression in F9 teratocarcinoma cells [11]. Together these studies show that CRABPs play an important role in mediating *at*RA actions.

*at*RA is eliminated mainly by oxidative metabolism [1,12,13]. Cytochrome P450 enzymes of the 26 family (CYP26s) are believed to be mainly responsible for metabolism of *at*RA during development and adult life [12–16]. Although CYP26A1 and CYP26B1 do metabolize *at*RA efficiently in the absence of CRABPs [17,18], it has also been shown that *at*RA bound to CRABP-I is metabolized[19,20], and CRABP-I increases the efficiency of *at*RA catabolism [11]. Since *at*RA binds both CRABP-I and CRABP-II tightly (K_d 0.06 and 0.13 nM, respectively) [8], it is expected that when CRABPs are expressed, no free *at*RA exists within the cell. Hence, the free-drug hypothesis, which states that drug binding to pharmacological receptors and metabolic enzymes is driven by free drug in solution and drug bound to other proteins is unavailable for receptor binding, suggests that *at*RA is either not available for metabolism or metabolism is greatly reduced in the presence of CRABPs. Despite this, in F9 teratocarcinoma cells, increased metabolism of *at*RA was observed when CRABP-I expression increased [11]. Similarly, in rat testes microsomes, that likely express CYP26B1, the depletion of *at*RA in the presence and absence of CRABP was identical, but the overall metabolite formation was decreased with increasing CRABP-I [19,20]. Taken together this data suggests that the free drug hypothesis is not applicable for *at*RA metabolism, and the CRABP(s) deliver *at*RA directly to the CYP enzymes responsible for *at*RA metabolism without a requirement of free *at*RA in cytosol. However, direct interaction between CRABPs and CYP26 enzymes has not been demonstrated, and the identity of the CRABPs and/or CYPs involved in the targeted metabolism of *at*RA have not been characterized. In addition, direct channeling of a substrate from a cytosolic carrier protein to any specific membrane bound mammalian CYP enzyme has not been previously demonstrated. The aim of this study was to determine whether CRABPs directly channel *at*RA to CYP26B1 and whether the CRABPs and CYP26B1 interact with each other.

Materials and Methods

Chemicals and Reagents

Ammonium acetate was obtained from Mallinckrodt Baker (Phillipsburg, NJ), and LC/MS-grade methanol, acetonitrile (ACN) and ethyl acetate from Fisher Scientific (Fairlawn, NJ). CYP3A4 and CYP2C8 Supersomes™ coexpressed with cytochrome P450 reductase and cytochrome b5 were purchased from BD Biosciences (Woburn, MO). 4-OH-RA was synthesized as previously described [21]. *at*RA and NADPH were purchased from Sigma Aldrich (St. Louis, MO). *at*RA-d_5 and 4-oxo-13-*cis*-RA-d_3 were obtained from Toronto Research Chemicals (North York, ON).

Enzyme expression

Recombinant CYP26B1 was expressed in Sf-9 cells using the baculovirus system and microsomes were prepared as described previously [18]. P450 reductase was expressed in *E. Coli* and purified as described previously [22]. For incubations P450 reductase was added to the membrane preparations as previously described [18]. The CRABP expression vectors

were provided by Dr. Noa Noy (Case Western University) and the CRABP-I and CRABP-II were expressed in *E. Coli* and purified as described previously [3] and the CRABP concentration was determined by BCA assay, UV absorbance and fluorescence titration. The binding of *at*RA was confirmed by fluorescence titration as described [8]. Holo-CRABPs were generated by mixing equal concentrations of *at*RA and CRABPs and the complete 1:1 binding was verified by fluorescence titration and UV spectroscopy.

Formation of 4-OH-RA by P450s in the presence and absence of CRABP-I and CRABP-II

Five pmol/mL of CYP3A4 and CYP2C8 were pre-incubated in 0.5 mL for 3 min with 1 mM NADPH and the reactions were initiated by addition of 1 μM holo-CRABP-I, holo-CRABP-II or *at*RA. The reaction was quenched after 10 minutes by the addition of an equal volume of acetonitrile followed by 5 mL ethyl acetate containing 30 pmol 4-oxo-RA-d_3 as internal standard. For CYP26B1, 5 pmol/mL P450 and 10 pmol/mL P450 reductase were preincubated with 1 mM NADPH for 3 minutes and the reaction was initiated by adding 50 nM *at*RA alone or 50 nM holo-CRABP-I or holo-CRABP-II. The reactions were quenched after 5 min with an equal volume of acetonitrile followed by the addition of 5 mL ethyl acetate and 30 pmol 4-oxo-RA-d_3. Following a liquid-liquid extraction, samples were dried under nitrogen and resuspended in 100 μL acetonitrile. 4-OH-RA formation was analyzed by LC-MS/MS as previously described [21]. All incubations were conducted in triplicate.

Effect of CRABP-I and CRABP-II on CYP26B1 mediated 4-OH-RA formation

The effect of CRABP-I and CRABP-II on the K_m and k_{cat} of 4-OH-RA formation by CYP26B1 was determined using 5 pmol/mL CYP26B1 with 10 pmol/mL P450 reductase in 0.5 mL incubations. The incubations were conducted with eight different concentrations (3.9 – 500 nM) of holo-CRABP-I, holo-CRABP-II or free *at*RA or *at*RA in the presence of albumin (20 mg/mL) and 4-OH-RA formation was measured. *at*RA was prebound to CRABP in a 1:1 ratio. CYP26B1 was preincubated with 1 mM NADPH for 3 min at 37°C and reactions were initiated with substrate. Following a 5 min incubation at 37°C, the reaction was quenched with an equal volume of acetonitrile, internal standard added (100 pmol 4-oxo-RA-d_3) and 4-OH-RA was extracted with 5 mL ethyl acetate. The concentration of 4-OH-RA was determined by LC-MS/MS analysis as previously described (21).

The inhibition of CYP26B1 by excess apo-CRABP was determined using 5 pmol/mL CYP26B1 and 10 pmol/mL reductase in 0.5 mL incubations. CYP26B1 and reductase were preincubated 3 min at 37°C with 1 mM NADPH and CRABP-I or CRABP-II at 6 different concentrations (0 – 500 nM). The reaction was initiated with 50 nM *at*RA and incubated for 5 min at 37°C. The reaction was quenched with equal volume of acetonitrile, 100 pmol internal standard (4-oxo-RA-d_3) was added and 4-OH-RA was extracted with 5 mL ethyl acetate. The concentration of 4-OH-RA was determined by LC-MS/MS as previously described [21].

Isotope dilution experiments with holo-CRABP-I, holo-CRABP-II and free atRA

For isotope dilution, 10 pmol CYP26B1 with 100 pmol P450 reductase were incubated in 0.5 mL with 100 nM holo-CRABP-I or holo-CRABP-II (bound with either *at*RA or *at*RA-d_5) and 100 nM free *at*RA or *at*RA-d_5 for 2 minutes. The reaction was initiated by adding

simultaneously holo-CRABP and free *at*RA. The isotope dilution was conducted both using holo-CRABP-I and holo-CRABP-II with *at*RA and adding *at*RA-d_5 free in solution and using holo-CRABP-I and holo-CRABP-II with *at*RA-d_5 and adding *at*RA free in solution. Formation of 4-OH-RA and 4-OH-RA-d_4 was monitored by LC-MS/MS as previously described [18], and the ratio between labeled and unlabeled products calculated. To control for intrinsic isotope effect of 4-hydroxylation, *at*RA and *at*RA-d_5 (100 nM each) were co-incubated with CYP26B1 as described above in the absence of CRABP-I and CRABP-II and the formation of 4-OH-RA and 4-OH-RA-d_4 measured.

Determination of kinetic constants

The velocity (v) of 4-OH-RA formation at increasing concentrations of *at*RA or holo-CRABP as a substrate ($[RA]_T$) was fit to the equation:

$$v=\frac{E_T k_{\mathrm{cat}}[RA]_T}{K_m+[RA]_T} \quad (1)$$

where E_T, is the total CYP26B1 concentration, k_{cat} is the catalytic rate constant and K_m substrate affinity constant. The velocity of 4-OH-RA formation both as a function of increasing holo-CRABP (1:1 CRABP:*at*RA ratio) and increasing total CRABP (constant *at*RA concentration) was globally fit to the equation:

$$v=\frac{E_T k_{\mathrm{cat}}[RA]_u\left(1+\frac{\beta[BP]_u}{\alpha K_d}\right)}{K_m\left(1+\frac{[BP]_u}{K_i}\right)+[RA]_u\left(1+\frac{[BP]_u}{\alpha K_d}\right)} \quad (2)$$

where K_d is the affinity constant for *at*RA with apo-CRABP and K_i is the affinity constant for apo-CRABP with CYP26B1. The K_d values used for CRABP-I and CRABP-II were 0.062 nM and 0.14 nM, respectively as reported [8]. The values of free *at*RA in solution ($[RA]_u$) and free CRABP-I and CRABP-II ($[BP]_u$) in solution were estimated from total *at*RA and CRABP concentrations ($[RA]_T$ and $[BP]_T$, respectively) and the binding constants using the quadratic formula:

$$[RA]_u=\frac{\sqrt{([BP]_T-[RA]_T+K_d)^2+4K_d[RA]_T}-([BP]_T-[RA]_T+K_d)}{2} \quad (3)$$

$$[BP]_u=\frac{\sqrt{([RA]_T-[BP]_T+K_d)^2+4K_d[BP]_T}-([RA]_T-[BP]_T+K_d)}{2} \quad (4)$$

For comparison the formation kinetics of 4-OH-RA from *at*RA by CYP26B1 in the presence of CRABP-I and CRABP-II and in the absence of any protein-protein interaction between

CYP26B1 and CRABPs (the free drug hypothesis model) was simulated using equation (5) and the same E_T, k_{cat} and K_m values as described above.

$$v = \frac{E_T k_{cat} [RA]_u}{K_m + [RA]_u} \quad (5)$$

The $[RA]_u$ was calculated by Equation 3.

All parameters were estimated via nonlinear regression using Solver for Microsoft Excel (Frontline Systems, Incline Village, NV, USA). All statistical analyses were conducted using GraphPad v.5 (La Jolla, CA, USA).

Results

CRABP-I and CRABP-II deliver *at*RA to CYP26B1 but not to CYP2C8 and CYP3A4

To determine whether CRABP-I or CRABP-II affect the metabolism of *at*RA by CYP enzymes, *at*RA was incubated with CYP26B1, CYP2C8 and CYP3A4 in the presence and absence of CRABP-I and CRABP-II. CYP26B1, CYP2C8 and CYP3A4 have all been shown previously to metabolize *at*RA to 4-OH-RA with K_m values of 19 nM, 13 μM and 19 μM, respectively [18,23], and formation of 4-OH-RA was detected with each CYP in this study as well (Figure 1A). In the presence of CRABP-I, the formation rate of 4-OH-RA by CYP26B1 was decreased by 52% ($p<0.001$) whereas formation of 4-OH-RA by CYP3A4 and CYP2C8 was decreased by 98% (Figure 1A). In the presence of CRABP-II the formation rate of 4-OH-RA by CYP26B1 was decreased by 28% ($p<0.001$). The formation of 4-OH-RA by CYP3A4 and CYP2C8 was reduced by 99% in the presence of CRABP-II compared to free *at*RA (Figure 1A). These data suggest that CRABP-I and CRABP-II target *at*RA selectively for metabolism by CYP26B1 and prevent the metabolism of *at*RA by other CYP enzymes. The fact that metabolism by CYP3A4 and CYP2C8 was effectively abolished in the presence of CRABP-I and CRABP-II demonstrates that *at*RA is extensively bound to CRABPs (effective sink in the absence of a receiving enzyme) in the incubations. Based on this data CRABPs minimize the presence of free *at*RA in solution and prevent the availability of free *at*RA for metabolism via partitioning to lipid and membrane access to the CYP. As such, these observations indicate that CRABP-I and CRABP-II deliver *at*RA directly to CYP26B1.

CYP26B1 accepts holo-CRABPs and free *at*RA as substrates

Previous research has shown that CYP26B1 can obtain free *at*RA in solution as a substrate and presence of CRABP-I or CRABP-II is not required for *at*RA turnover or delivery to CYP26B1 *in vitro* [18]. In addition, since *at*RA is a high affinity ligand of CYP26B1 it is expected that any *at*RA that dissociates from CRABPs during the incubation will bind to CYP26B1. Hence, isotope dilution experiments were conducted to determine whether CYP26B1 obtains *at*RA as a substrate directly from CRABP-I and CRABP-II or whether ligand release to solution is required prior to binding to CYP26B1. When *at*RA and *at*RA-d_5 were incubated with CYP26B1, an isotope effect was observed and metabolism of the *at*RA-d_5 was 50% slower than that of *at*RA (4-OH-RA/4-OH-RA-d_4 ratio was 1.5) likely due to

the fact that the deuterium atoms in the labeled compound are located at C-4 and C-18 of *at*RA, the sites of metabolism. When *at*RA was bound with CRABP-I or CRABP-II and coincubated with *at*RA-d_5 free in solution, 4-OH-RA/4-OH-RA-d_4 ratios of 1.69 ± 0.24 and 2.58 ± 0.51 were observed with CRABP-I and CRABP-II respectively. The 4-OH-RA/4-OH-RA-d_4 ratios were 1.37 ± 0.14 and 0.81 ± 0.02 when *at*RA-d_5 was bound with the CRABP-I and CRABP-II, respectively, and *at*RA was free in solution. When the isotope dilution values were corrected for the isotope effect of faster metabolism of unlabeled than labeled *at*RA, the ratio between the products arising from bound and free *at*RA was 1.1 for CRABP-I and 1.8 for CRABP-II. In the absence of channeling, CYP26B1 should obtain the substrate from free in solution and the ratio of products arising from bound *at*RA over free *at*RA should approach zero. A ratio of 1 indicates that channeling does occur but the CYP does not differentiate between the holo-CRABP and *at*RA free in solution as substrates. A ratio of 1.8 obtained with CRABP-II suggests that the channeling of *at*RA from CRABP-II to CYP26B1 is preferred over delivery of *at*RA to CYP26B1 from solution or the lipid bilayer. Taken together the isotope dilution data suggests that holo-CRABPs interact directly with CYP26B1 via protein-protein interaction that results in *at*RA delivery to CYP26B1.

Holo-CRABPs have lower apparent K_m and k_{cat} than *at*RA with CYP26B1

To further evaluate the metabolism of *at*RA from holo-CRABP-I and holo-CRABP-II, the apparent Michaelis-Menten kinetics for holo-CRABP-I and holo-CRABP-II were determined in comparison to *at*RA free in solution (Figure 1B). The apparent K_m and k_{cat} values (Figure 1B) were both decreased for holo-CRABP-I and holo-CRABP-II ($p<0.0001$) when compared to free *at*RA in solution. Formation of 4-OH-RA from free *at*RA had a K_m of 64.6 ± 10.3 nM and a k_{cat} of 0.45 ± 0.02 pmol/min/pmol P450. Formation of 4-OH-RA from holo-CRABP-I had a k_{cat} of 0.17 ± 0.006 pmol/min/pmol and an apparent K_m of 21.7 ± 2.9 nM. With holo-CRABP-II the k_{cat} value was 0.28 ± 0.01 pmol/min/pmol and apparent K_m 24.3 ± 4.2 nM. If the unbound fractions for *at*RA were accounted for in the incubations the unbound apparent K_m was decreased 27-fold in the presence of CRABP-I and 18-fold in the presence of CRABP-II in comparison to *at*RA. As a control of the effect of a nonspecific protein sink to *at*RA hydroxylation by CYP26B1, albumin was added into the incubations at a concentration of 20 mg/mL. At this albumin concentration the unbound fraction of *at*RA was 0.01. As expected based on the free drug hypothesis, presence of albumin decreased *at*RA 4-hydroxylation rate significantly ($p<0.05$ for 250 nM and 500 nM *at*RA, paired t-test) in comparison to *at*RA free in solution (Figure 1B) demonstrating that free drug hypothesis accurately describes *at*RA metabolism in the presence of albumin. The higher product formation velocity from holo-CRABPs in comparison to albumin bound *at*RA further supports a direct protein-protein interaction between CRABPs and CYP26B1. The fact that the apparent K_m was decreased for holo-CRABPs in comparison to free *at*RA is in contrast to the free drug hypothesis and can only be explained via direct delivery of *at*RA from CRABPs to CYP26B1.

Apo-CRABPs inhibit CYP26B1 mediated metabolism of *at*RA

The decreased K_{cat} value of 4-OH-RA formation from holo-CRABP-I and holo-CRABP-II suggested that CRABPs act as noncompetitive inhibitors of CYP26B1. To test whether CRABP-I and CRABP-II are inhibitors of CYP26B1, increasing concentrations of apo-

CRABPs were added to incubations of holo-CRABPs. Increasing concentrations of apo-CRABPs inhibited the formation of 4-OH-RA in a concentration dependent manner (Figure 1C). holo-CRABP-I and holo-CRABP-II also inhibited the metabolism of 9-*cis*RA by CYP26B1 (data not shown). Together these data showt that apo-CRABP-I and apo-CRABP-II interact directly with CYP26B1 and inhibit *at*RA metabolism. Such direct inhibitory interaction between apo-CRABPs and CYP26B1 may be important in the control of cellular concentrations of *at*RA by allowing fine tuning CYP26B1 activity based on *at*RA to CRABP concentration ratio.

Kinetic Model of CRABP-CYP26B1 interactions

To further establish the kinetics of the interaction between CYP26B1 and CRABP-I and CRABP-II, a kinetic scheme and a velocity equation of the interactions was generated (Figure 2A), The model was globally fitted to the product formation data obtained in the presence and absence of CRABPs to obtain kinetic parameters (Figure 2).. Based on the kinetic parameter estimates, apo-CRABP-I and apo-CRABP-II inhibit CYP26B1 within biologically relevant concentrations (~50 nM) and the affinity of holo-CRABP-I (αK_m=0.024 nM) and holo-CRABP-II (αK_m=0.059 nM) with CYP26B1 is higher than that of *at*RA free in solution. However, the kinetic analysis also suggests that the catalytic rates of *at*RA hydroxylation are decreased by the presence of CRABP-I and CRABP-II. The formation of 4OH-RA by CYP26B1 in the presence of CRABPs was then simulated using the traditional free drug hypothesis model with sequestration of *at*RA by CRABPs and using the complete protein-protein interaction model (Figure 2B and C).

DISCUSSION

For several decades the role of the individual CRABPs in mediating *at*RA metabolism has been unclear. The CRABPs are highly conserved across species and the expression patterns of CRABP-I and CRABP-II are distinctly different both during embryonic development and adult life [6]. It has been well established that CRABP-II facilitates transcriptional activation of RARs by *at*RA [7]. However, the role of CRABP-I has not been as well determined in various tissues with different expression of *at*RA metabolizing enzymes, and the overall effects of both CRABPs on metabolism of *at*RA have not been established. In fact, the effect of CRABP-II on *at*RA metabolism has not been previously reported in any system. In F9 cells CRABP-I expression has been shown to result in lower sensitivity of the cells to *at*RA [21], and CRABP-I silencing experiments in F9 cells showed that increasing expression of CRABP-I increased the metabolism of *at*RA and decreased the cellular half-life of *at*RA [11]. In rat testes microsomes, *at*RA bound to CRABP-I was shown to be metabolized with greater efficiency than free *at*RA indicating a direct role of CRABP-I in *at*RA metabolism [19]. It is important to note that in all of these systems the enzymes responsible for *at*RA metabolism are not known. This study shows that both CRABP-I and CRABP-II interact specifically with CYP26B1. In addition, the data shows that while apo-CRABP-I and apo-CRABP-II inhibit the metabolism of *at*RA by CYP26B1, holo-CRABPs specifically deliver *at*RA to CYP26B1 and the affinity of holo-CRABPs to CYP26B1 is significantly greater than that of free *at*RA. This increase in the affinity of holo-CRABPs to CYP26B1 in comparison to the decreased k_{cat} leads to an increased intrinsic clearance (k_{cat}/K_m) of holo-

CRABPs, potentially explaining the observed faster metabolic rates of *at*RA in cells with higher CRABP expression and the higher metabolic efficiency in rat testes microsomes in the presence of CRABP-I.

The observed product formation data with CRABPs and CYP26B1 can only be explained via substrate channeling and protein-protein interactions, and as shown in Figure 2, CRABPs simply sequestering free *at*RA from solution would result in distinctly different product formation kinetics with a higher apparent K_m (unchanged unbound K_m) but unchanged k_{cat}. The observed protein-protein interactions appear to be substrate and CYP26 specific. Based on preliminary studies conducted by us (data not shown), CRABP-I and CRABP-II also interact with CYP26A1 and CYP26C1, the other members of the CYP26 family, in a similar manner. However, as shown here with CYP3A4 and CYP2C8, in the absence of CYP26s CRABPs sequester *at*RA from solution consistent with the free drug hypothesis. Similarly, in the presence of significant albumin binding, *at*RA is sequestered from metabolism despite its high affinity to CYP26B1 demonstrating the specificity of the CRABP-CYP26 interaction. Finally, the substrate channeling appears to be specific for *at*RA as a substrate as CRABP-I binding has been shown to prevent the metabolism of 4OH-*at*RA and 4oxo-*at*RA in rat testes microsomes [20] despite the fact that both metabolites bind CRABP-s with similar affinity as *at*RA[19] and are efficiently metabolized by CYP26B1 [18, 21].

The fact that both CRABPs deliver *at*RA to CYP26B1 is surprising, as only CRABP-II has been shown to deliver *at*RA to RAR and localize to the nucleus in the presence of *at*RA [7,8]. It was expected that only CRABP-I would deliver *at*RA to CYP26B1 in the ER for metabolism. However, all the data presented here suggest that both CRABP-I and CRABP-II interact with CYP26B1 and channel *at*RA to CYP26B1 although it is possible that post-translational modifications of CRABPs alter the interactions between CRABPs and CYP26B1. Our data is consistent with the reported localization data of CRABP-II [10] in which CRABP-II is associated with the ER and SUMOylation upon *at*RA binding in cells results in translocation of CRABP-II into the nucleus followed by export of CRABP-II from the nucleus and re-association of CRABP-II with the ER. Our data suggests that CRABP-II associates with the ER through tight interactions with CYP26B1.

The inhibition of CYP26B1 by CRABPs is in agreement with the previous data reported in rat testes microsomes in which the total amount of radioactive *at*RA metabolites decreased with increasing apo-CRABP-I concentrations [19]. Interestingly, the data shown here suggests that both CRABP-I and CRABP-II play an important role in regulating the metabolic rate of *at*RA and that the ratio of apo-CRABP to holo-CRABP for both CRABPs influences the metabolism of *at*RA. The inhibition of CYP26B1 by apo-CRABPs is biologically plausible as it allows the ratio of apo to holo-CRABP in the cell to mediate the metabolic rate of *at*RA. According to this model, if cellular *at*RA concentrations are low (lower than CRABP concentrations) apo-CRABP will inhibit the metabolism of *at*RA and aid in combatting *at*RA deficiency. Yet, in situations in which the cell is exposed to excess *at*RA (i.e. *at*RA concentrations exceed CRABP concentrations) holoCRABP will facilitate *at*RA metabolism. It is unlikely that the CRABPs fit within the active site of CYP26B1 and therefore the inhibition of CYP26B1 by CRABPs is expected to be due to CRABPs binding or interacting with a surface site of CYP26B1. It is possible that the CRABPs bind at an

allosteric site decreasing catalytic activity but more studies are required to identify the interacting residues and subsequent possible conformational changes in the CYP26B1 protein.

At present there are no reports of a specific mammalian membrane bound CYP accepting a substrate directly from a soluble carrier protein. However, a crystal structure of a bacterial CYP107H1 in complex with the fatty acid linked to acyl carrier protein has been reported [24], providing structural insight how these carrier protein- CYP interactions may occur. Mammalian CYP enzymes are generally believed to gain access to their substrates via the lipid bilayer in which they are embedded, or through a substrate access channel facing the cytosol. It would be possible that CRABPs deliver *at*RA into the lipid membrane and not directly to the CYP. However, if this was the case, the delivery of the substrate would not be expected to be CYP-enzyme specific as shown here with the lack of holoCRABP metabolism by CYP3A4 and CYP2C8. Similarly, *at*RA can partition to the lipid bilayer in the presence of albumin but the metabolism of *at*RA in the presence of albumin was significantly less than in the presence of CRABPs supporting a specific substrate delivery by CRABPs to CYP26B1 via cytosolic access channel. Direct inhibition of CYP3A4 and CYP2C8 by CRABPs was overruled in an experiment using midazolam as a CYP3A4 substrate. CRABP-I and CRABP-II had no effect on CYP3A4 mediated hydroxylation of midazolam, a substrate that does not bind to CRABP (data not shown). Hence both the substrate delivery and the inhibitory effect of CRABPs was specific to the CYP enzyme considered as the endogenous *at*RA hydroxylase (CYP26B1).

In conclusion, this study shows that soluble cytosolic carrier proteins such as CRABPs can interact with ER membrane bound CYP enzymes and mediate oxidative metabolism of CYP substrates. This suggests that such interactions with other soluble carrier proteins and CYP enzymes may also occur and mediate the metabolism of steroids, fatty acids and other fat soluble vitamins. Similar to the CRABP-II- RAR interaction, the fatty acid binding proteins (FABPs) have been shown to interact with PPAR in the nucleus [9] and it is possible that FABPs also interact with CYP enzymes in the ER. This may have broad effects on the metabolism of fat soluble substrates by CYPs in the liver and other tissues.

Acknowledgments

This work was supported by NIH grants R01-GM081569 and R01-GM111772 and by a pilot grant from the Royalty Research Fund of University of Washington. The authors wish to thank Dr Sumit Goswami, Dr. Jayne E. Thatcher and Elijah Weber for their assistance in enzyme expression, preparation of microsomes and conduct of preliminary experiments, Dr Noa Noy for providing the CRABP expression vectors and Dr Wendel L Nelson for helpful discussions during this work.

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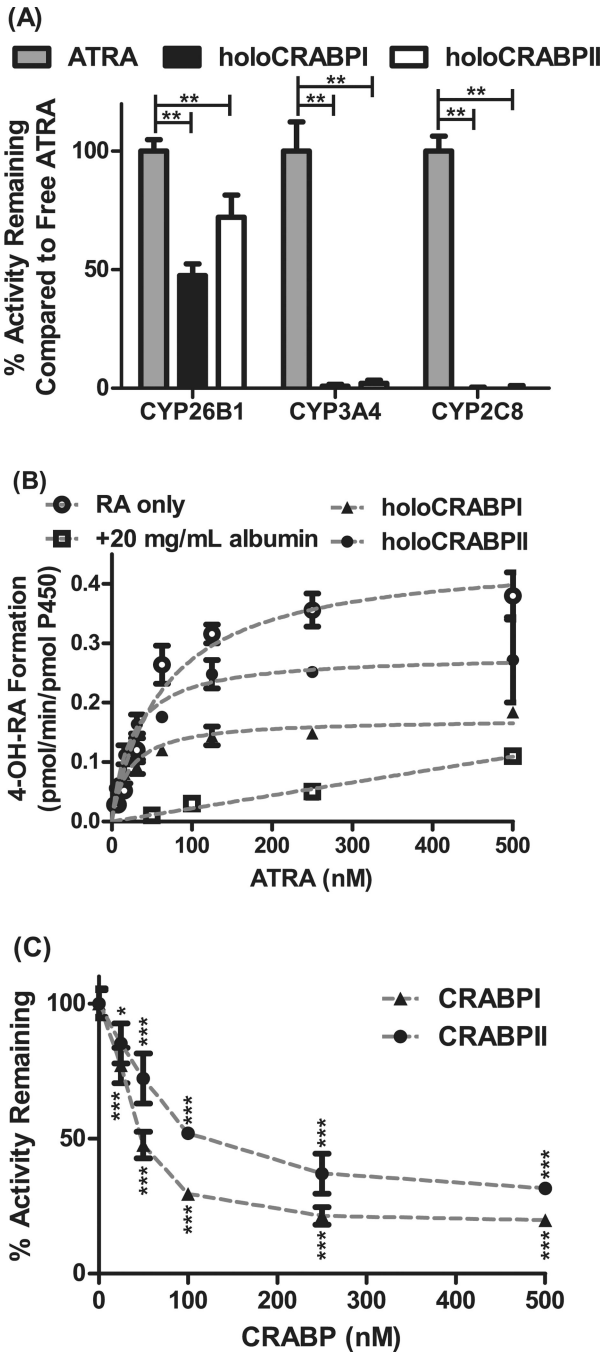
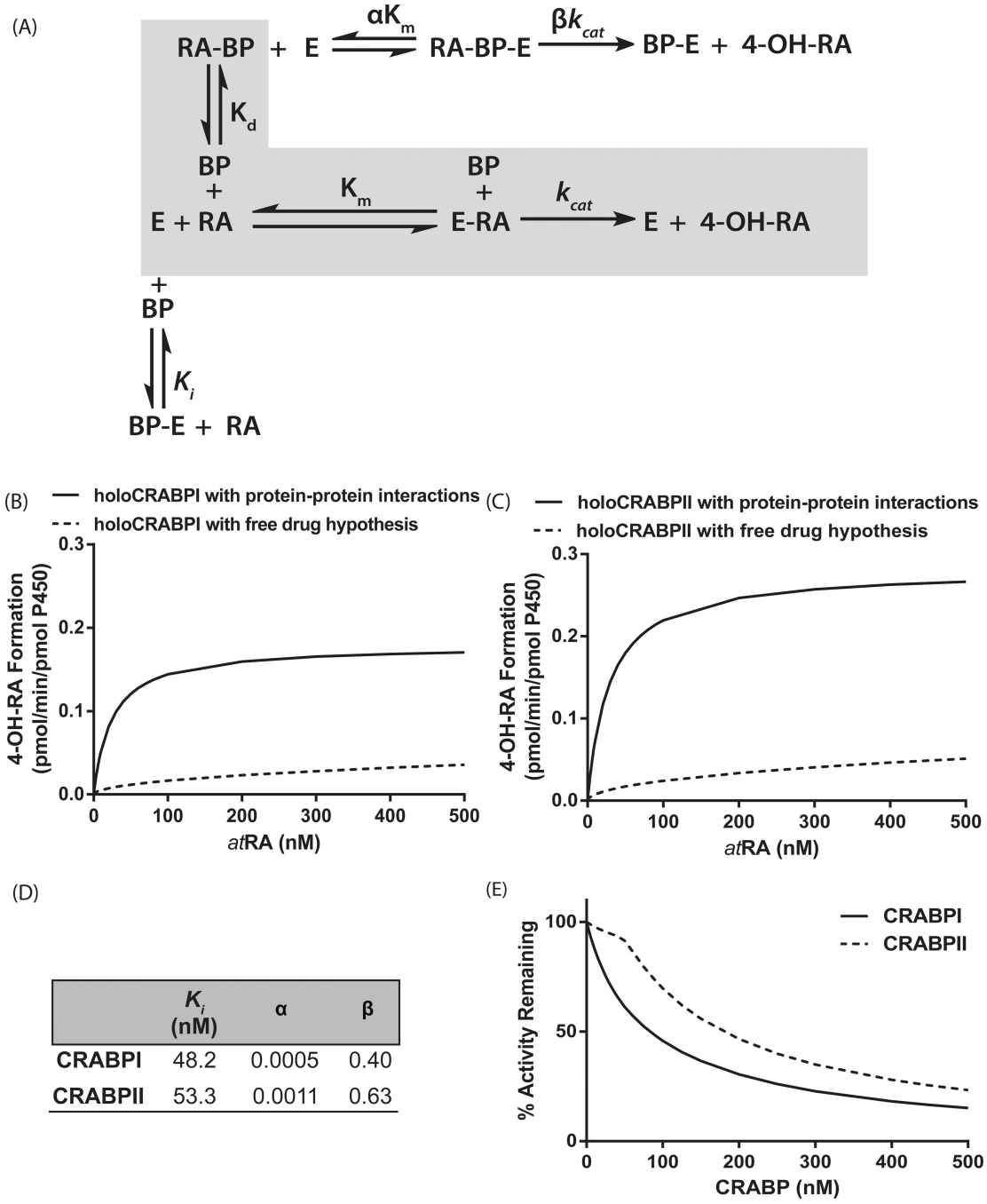


Figure 1. Effect of CRABP-I and CRABP-II on *at*RA metabolism by CYP26B1

Panel A shows the 4-OH-RA formation from *at*RA by CYP26B1, CYP3A4 and CYP2C8 in the presence and absence of CRABP-I and CRABP-II. While *at*RA is metabolized efficiently by CYP26B1 in the presence of CRABP-I and CRABP-II, metabolism by CYP3A4 and CYP2C8 is completely prevented in the presence of CRABPs. (**p<0.001, two-way ANOVA with Bonferroni post-test) Panel B shows the determination of K_m and k_{cat} values for 4-OH-RA formation by CYP26B1 when holo-CRABP-I, holo-CRABP-II, free *at*RA or *at*RA bound with albumin is used as a substrate. The apparent K_m and k_{cat}

values were 64.6 ± 10.3 nM and 0.45 ± 0.02 pmol/min/pmol CYP for free *at*RA, 21.7 ± 2.9 nM and 0.17 ± 0.006 pmol/min/pmol P450 for holoCRABPI, and 24.3 ± 4.2 nM and 0.28 ± 0.01 pmol/min/pmol CYP for holoCRABPII. Panel C shows the inhibition of CYP26B1 mediated 4-OH-RA formation by excess apo-CRABP. *at*RA concentration was 50 nM in this experiment. (*p<0.05, ***p<0.0001, one-way ANOVA with Dunnett post-hoc test).



	K_i (nM)	α	β
CRABPI	48.2	0.0005	0.40
CRABPII	53.3	0.0011	0.63

Figure 2.

Comparison of the models of the metabolism of *at*RA by CYP26B1 according to the free drug hypothesis and incorporating the protein–protein interactions between CRABPs and CYP26B1. Panel (A) shows the kinetic scheme of the interactions between CRABPs (BP), CYP26B1 (E), and *at*RA. The shaded gray area indicates the kinetic scheme for the free drug hypothesis, while the complete scheme shows the effect of substrate channeling and protein–protein interactions. Panels (B) and (C) show the simulated product formation curves in the presence of CRABP-I (B) and CRABP-II (C) using either the kinetic

parameters obtained from a global fit to the data in Fig. 1B,C (solid lines, protein–protein interaction model) or using the independently measured kinetic constants in the absence of protein–protein interactions (dotted line, free drug hypothesis). The fitted kinetic constants for CRABP interactions with CYP26B1 according to the substrate channeling model are shown in panel (D). Panel (E) shows the simulated product formation curves in the presence of increasing concentrations of apo-CRABP using the kinetic constants from the global fit of the experimental data to the protein–protein interaction model. Using the fitted parameters shown in panel (D), velocity was calculated using Eqn (2) for a range of *at*RA and CRABP concentrations (0–500 nM) and plotted using GRAPHPAD PRISM v5. The simulations were conducted as described in Materials and methods section.