Differential Mechanisms of Retinoid Transfer from Cellular Retinol Binding Proteins Types I and II to Phospholipid Membranes

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Cellular retinol-binding proteins types I and II (CRBP-I and CRBP-II) are known to differentially facilitate retinoid metabolism by several membrane-associated enzymes. The mechanism of ligand transfer to phospholipid small unilamellar vesicles was compared in order to determine whether differences in ligand trafficking properties could underlie these functional differences. Unidirectional transfer of retinol from the CRBPs to membranes was monitored by following the increase in intrinsic protein fluorescence that occurs upon ligand dissociation. The results showed that ligand transfer of retinol from CRBP-I was >5-fold faster than transfer from CRBP-II. For both proteins, transfer of the other naturally occurring retinoid, retinaldehyde, was 4-5-fold faster than transfer of retinol. Rates of ligand transfer from CRBP-I to small unilamellar vesicles increased with increasing concentration of acceptor membrane and with the incorporation of the anionic lipids cardiolipin or phosphatidylycerine into membranes. In contrast, transfer from CRBP-II was unaffected by either membrane concentration or composition. Preincubation of anionic vesicles with CRBP-I was able to prevent cytochrome c, a peripheral membrane protein, from binding, whereas CRBP-II was ineffective. In addition, monolayer exclusion experiments demonstrated differences in the rate and magnitude of the CRBP interactions with phospholipid membranes. These results suggest that the mechanisms of ligand transfer from CRBP-I and CRBP-II to membranes are markedly different as follows: transfer from CRBP-I may involve and require effective collisional interactions with membranes, whereas a diffusional process primarily mediates transfer from CRBP-II. These differences may help account for their distinct functional roles in the modulation of intracellular retinoid metabolism.

Vitamin A is an essential micronutrient required for growth, differentiation, reproduction, and normal vision. The selective movement of retinoids throughout the body is a subject of intense interest due to these pleiotropic effects. Dietary vitamin A leaves the enterocyte on chylomicrons in the form of retinyl esters and is delivered to the liver in the chylomicron remnant. Hepatic processing results in either release of retinol bound to the extracellular retinol-binding protein (RBP)1 or storage instellate cells as retinyl esters. The released retinol is transported to target tissues bound to RBP where it is internalized and converted to its active forms. Within cells, cellular retinol-binding proteins (CRBPs) are thought to facilitate retinoid metabolism by presenting ligand to specific metabolic enzymes. The precise roles of extracellular and intracellular retinoid-binding proteins in ligand transport and targeting are nevertheless not defined.

It has been shown in vitro that retinoids are capable of partitioning into the hydrophobic phase of membranes and that transfer between lipid vesicles or to intracellular binding proteins can occur via diffusional processes (1, 2). The degree to which this spontaneous diffusional movement accounts for retinoid trafficking in a cellular context is controversial. An alternative theory suggests that the directed channeling of retinol to membrane lipid or protein compartments occurs via its specific binding proteins. For example, an early report that examined the kinetics of retinol transfer from RBP to liposomes suggested that retinol transfer occurred by collisional interactions between RBP and membranes (3). In addition, retinol entry into cells may involve uptake via a specific cell-surface RBP receptor. A recent study suggests that the RBP receptor and CRBP-I within cells are able to interact directly, resulting in a channeling of the hydrophobic ligand from the extracellular RBP to the intracellular CRBP-I (4).

Extensive studies directed at the functional role of the CRBPs have shown that particular forms of these binding proteins are also able to interact with specific metabolic enzymes. This family of binding proteins includes cellular retinol-binding protein (type I), distributed widely throughout the body, and cellular retinol-binding protein type II, restricted to the enterocyte and the neonatal hepatocyte (reviewed in Refs. 5 and 6). These two binding proteins have 56% sequence identity, they both bind retinol and retinaldehyde with high affinity, and their crystallographic structures are virtually superimposable (5, 6). Holo-CRBP-I and -CRBP-II are both able to serve as substrate for the esterification of retinol by the microsomal enzyme lecithin retinol acyltransferase (LRAT). Despite these similarities, apoCRBP-I has been shown to inhibit LRAT, whereas apocrBP-II does not (7). The ability of LRAT to differentiate between these structurally similar proteins and between the apo and holo forms implies that subtle conformational differences may dictate differential recognition of these binding proteins by enzymes and further suggests that functional differences between CRBP-I and CRBP-II may exist. Similar discrimination between apo and holo-CRBP-I has been

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‡ The abbreviations used are: RBP, retinol-binding protein; SUV, small unilamellar vesicles; CRBP, cellular retinol-binding proteins; EPC, egg phosphatidylcholine; EPE, egg phosphatidylethanolamine; NBD, N-(7-nitro-2,1,3-benzoxadiazol-4-yl); dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DPE, dansyl phosphatidyl ethanolamine; LRAT, lecithin retinol acyltransferase; FABP, fatty acid-binding protein; CL, cardiolipin; PS, phosphatidylserine; mN, millinewton.
observed with the microsomal NADP-dependent retinol dehydrogenase (8). The molecular determinants by which enzymes discriminate between binding proteins are as yet unknown.

Several of the enzymes involved in cellular vitamin A metabolism are membrane-associated. It is therefore possible that in addition to interacting with the enzymes themselves, the binding proteins may also be interacting with a lipid component in the bilayer. However, only limited information on the role of these proteins in trafficking retinoids from and/or between membranes is available. Previous studies have demonstrated that partitioning of retinol from CRBP-I to model membranes can occur (9, 10). Transfer from CRBP-II to membranes has also been reported, but kinetic measurements were not made (10). Here, using changes in intrinsic protein tryptophan fluorescence, we are able to directly examine and compare the kinetics and mechanism of transfer of retinol from CRBP-I and CRBP-II to phospholipid membranes. We demonstrate that the two proteins transfer ligand to model membranes at significantly different rates and via distinctly different mechanisms. Transfer from CRBP-I may involve and require effective colloidal interactions with membranes, whereas a diffusion process primarily mediates transfer from CRBP-II. In addition, two assays were employed to examine directly the interaction of CRBPs with model membranes. In both systems the two proteins interacted with membranes to markedly different degrees, and these differences substantiate the disparities observed in the retinoid transfer assays. The differences in membrane interaction may help account for the differential recognition of CRBP-I and CRBP-II by metabolic membrane-bound enzymes and may relate to differences in the selective trafficking of retinol in the cell.

EXPERIMENTAL PROCEDURES

Materials—All-trans-retinol, all-trans-retinaldehyde, and cytochrome c (type VI, horse heart) were from Sigma. Egg phosphatidylcholine (EPC), egg phosphatidylethanolamine (EPE), N'(7-nitro-2,3-benzoxadiazol-4-yl) (NBD) egg phosphatidylethanolamine, brain phosphatidylserine (PS), bovine heart cardiolipin (CL), and N(5-dimethylaminocyclononaphthalene-1-sulfonylethanolamine-3-phosphate) (DPE) were obtained from Avanti Polar Lipids (Alabaster, AL). All other reagents were of the highest grade available. Rat apoCRBP-I and CRBP-II were purified using a recombinant expression system as described previously (11). Holoprotein was prepared by addition of equimolar ligand (in ethanol, final ethanol < 0.01% v/v), and saturation was monitored by following the increase in retinol fluorescence (λex 350 nm).

Vesicle Preparation—Small unilamellar vesicles (SUVs) were prepared by sonication and ultracentrifugation as described (12). The typical SUVs used in these studies were composed of 100% EPC. To examine the effect of membrane charge, 10 mol % of either PS or CL was incorporated in place of an equimolar amount of EPC. The SUVs were prepared in 40 mM Tris-Cl, 100 mM NaCl, pH 7.4. For CL-containing SUVs the buffer included 1 mM EDTA and for the studies involving changes in ionic strength the vesicles were prepared in 40 mM Tris-Cl, pH 7.4. For the cytochrome c displacement studies, the vesicles were composed of 64 mol % EPC, 10 mol % DPE, 25 mol % CL, and 1 mol % DPE and were prepared in 20 mM Tris-Cl, 0.1 mM EDTA, pH 7.4. The concentration of phospholipid was determined by quantitation of total inorganic phosphate (13), and concentrations of vesicles containing CL were corrected for the two phosphate groups per CL molecule.

Measurement of Equilibrium Partitioning—The molar equilibrium distribution of retinol between CRBPs and EPC SUVs (Kp) was estimated by quantifying the increase in intrinsic tryptophan fluorescence of the proteins upon release of ligand to SUVs (14) as shown in Equation 1.

\[
K_p = \frac{F_n}{F_i} / \frac{F_n - F_i}{[SUV]} \quad \text{(Eq. 1)}
\]

where \(F_n\) and \(F_i\) are relative fluorescence intensities in the absence or presence of [SUV]. Equation 1 can be rewritten as Equation 2.

\[
1/\Delta F = (K_p)/(1/\Delta F_{max}) \text{mol EPC/mol CRBP} + 1/\Delta F_{max} \quad \text{(Eq. 2)}
\]

where \(\Delta F\) is the relative difference in fluorescence at a given molar ratio of lipid to protein, \(\Delta F_{max}\) is the maximal fluorescence intensity change, and \(K_p\) is the slope of the resultant line (15). Holo-CRBP-I or -CRBP-II (1 μM in 40 mM Tris-Cl, 100 mM NaCl, pH 7.4) was titrated with increasing amounts of EPC SUV, and partitioning was monitored by measuring the increase in tryptophan fluorescence (λem 290 nm) using an SLM-8000 spectrofluorometer. Relative fluorescence emission intensities at 340 nm were used in Equation 2 to calculate \(K_p\) values.

Transfer of Retinoids from CRBPs to Phospholipid Vesicles—The \(K_p\) values for CRBP-I and CRBP-II, estimated as above, were necessary to determine the minimum amount of lipid necessary to ensure a ≥1:1 ratio of donor:acceptor binding capacity in the retinol transfer assays so that unidirectional transfer rates could be assessed (16, 17). Thus, transfer from CRBP-I was examined at a lipid:protein molar ratio of ≥500:1, and transfer from CRBP-II was examined at a lipid:protein molar ratio of ≥100:1. By surpassing the \(K_p\) values, all transfer assays were done at lipid levels that ensured distribution of ligand to membranes, and therefore, rate comparisons between proteins are valid.

Transfer of retinol or retinaldehyde from CRBPs to SUVs was monitored by two different transfer assays. First, a fluorescence resonance energy transfer assay was used to monitor the transfer of retinol from CRBP-II to EPC vesicles containing the fluorescent quencher NBD-PC. Briefly, 2 μM holo-CRBP-II was mixed with increasing concentrations of SUVs, and a time-dependent decrease in retinol fluorescence (excitation 350 nm, emission filter 450 ± 35 nm) was observed upon transfer of retinol to the NBD-containing SUVs (9). Transfer from holo-CRBP-I to NBD-containing vesicles was not detectable using this method due to the large inner filter quenching of retinol fluorescence and the high lipid:protein molar ratio of 500:1. As a result, a second assay was developed to monitor changes in protein fluorescence upon transfer of the ligand from protein to membranes.

The protein fluorescence assay makes use of the decrease in intrinsic protein fluorescence of the CRBPs that occurs upon ligand binding (14). This quenching of holoprotein fluorescence is relieved when ligand is released from the binding site (9). Thus, upon mixing holoprotein (1 μM retinol-CRBP-I or 2 μM retinol-CRBP-II) with EPC SUVs, release of ligand from the protein is monitored via a time-dependent increase in protein fluorescence (excitation 287 nm, emission filter 355 ± 60 nm). For each condition used, a rate was obtained by averaging the values from ≥5 separate transfer data sets. An example of a single transfer data set is shown in Fig. 1. The molar excess of acceptor lipid necessary to ensure unidirectional transfer was determined by the partition coefficients estimated as described above, and the final ratios used are indicated in each figure legend.

For the transfer assays, proteins and lipids were typically in 40 mM Tris-Cl, 100 mM NaCl, pH 7.4, and transfer was monitored at 25 °C unless noted otherwise. For studies of the effects of ionic strength, NaCl was added to the SUV and protein samples prior to mixing. Both the
Retinoid Transfer from CRBP-I and -II to Membranes

RESULTS

Equilibrium Partition Coefficients—In order to examine the kinetics of unidirectional retinol transfer to phospholipid vesicles, we first determined the equilibrium partition coefficients for retinol between CRBP-I or CRBP-II relative to model phospholipid vesicles as described under “Experimental Procedures.” As expected, for both CRBP-I and CRBP-II, fluorescence emission at 320 nm increased upon addition of SUV; a representative experiment is shown in Fig. 2. Titrating in increasing concentrations of SUV and transforming the fluorescence data (inset, Fig. 2) resulted in calculated $K_p$ values of $300 \pm 50$ for CRBP-I and $24 \pm 9$ for CRBP-II. These apparent partition coefficients suggest that CRBP-I has a higher retinol binding affinity than does CRBP-II. Previous analysis of the relative affinities of these two proteins for retinol have yielded conflicting results. Fluorometric titration studies indicated that CRBP-I and CRBP-II have similar $K_d$ values for retinol ($10 \text{ nm}$) (22, 23), whereas NMR analyses have suggested that CRBP-I may have as much as 100-fold greater affinity for retinol than does CRBP-II (11). The present partition data indicate that CRBP-I and CRBP-II differ in their affinity for retinol by approximately an order of magnitude, in that 12-fold more SUV is required to obtain equivalent ligand dissociation from CRBP-I relative to CRBP-II.

Transfer of Retinol from CRBPs to Membranes—Transfer of retinol from CRBP-I and CRBP-II to SUVs was examined using two different fluorometric assays as described under “Experimental Procedures.” In the first method, transfer of ligand from the protein to the NBD-SUVs was monitored by the time-dependent decrease in retinol fluorescence caused by NBD quenching. As the $K_p$ for CRBP-I dictated a minimum of 300-fold molar excess lipid to ensure unidirectional transfer, inner filter quenching of retinol fluorescence by the NBD fluorophore precluded the use of this assay with holo-CRBP-I. However, holo-CRBP-II did not require such high lipid:protein ratios, and filter quenching of retinol fluorescence by the NBD fluorophore permitted the use of this assay with holo-CRBP-I. However, holo-CRBP-II did not require such high lipid:protein ratios, and the NBD-based transfer assay yielded transfer rates of $0.08 \pm 0.01 \text{ s}^{-1}$ using a 100-fold excess of lipid relative to protein and $0.07 \pm 0.01 \text{ s}^{-1}$ using 200-fold excess lipid.

In the second method, transfer of ligand to membranes was monitored by increases in binding protein tryptophan fluorescence, in the absence of any additional fluorophore. As the absolute amount of lipid did not interfere with tryptophan fluorescence measurements, mechanisms and rates of transfer of retinol from both CRBP-I and CRBP-II could be compared directly. The results showed that transfer rates for retinol from CRBP-I to SUVs were, at minimum, 6-fold faster than rates from CRBP-II (0.46 $\pm 0.07 \text{ s}^{-1}$ versus $0.08 \pm 0.005 \text{ s}^{-1}$, Fig. 3). Importantly, the results for CRBP-II using this tryptophan fluorescence assay were virtually identical to those obtained with the retinol-NBD energy transfer method (at a 100-fold molar excess lipid, $k = 0.08 \pm 0.01 \text{ s}^{-1}$, and at 200-fold molar excess lipid, $k = 0.07 \pm 0.004 \text{ s}^{-1}$, Fig. 3). Thus, rates of retinol...
transfer from CRBP-II were identical when monitored by either the quenching of retinol fluorescence or the increase in protein fluorescence.

Effect of Vesicle Concentration and Charge on Retinol Transfer to Membranes—The transfer of a hydrophobic ligand from a protein to a membrane can occur via several different mechanisms. There can be effective collisional interactions between protein and membrane lipids, resulting in ligand transfer. There can be release of the ligand to the aqueous milieu, diffusion through the aqueous phase and then association onto the membrane, and finally there can be a combination of both collisional and diffusional events. In order to discern between these transfer mechanisms, the concentration and charge of acceptor vesicles were varied. Fig. 3 shows the results obtained when a constant concentration of holoprotein was mixed with increasing concentrations of EPC-SUV. Increasing the concentration of acceptor membrane did not alter the rate of transfer of retinol from holo-CRBP-II, whereas an increase in acceptor lipid increased the transfer rate from holo-CRBP-II. These results suggest that transfer of retinol from CRBP-I includes effective collisions between protein and lipid, whereas transfer from CRBP-II does not appear to involve such interactions.

The potential differences in retinol transfer mechanisms were further explored by changing the surface charge density of the acceptor vesicles, since alterations in acceptor membrane properties can only influence the ligand transfer rate in the case of a collisional mechanism (24, 25). Indeed, increasing the net negative charge on phospholipid vesicles increased the retinol transfer rate from CRBP-I but not from CRBP-II (Fig. 4). Transfer from CRBP-I to vesicles containing 10 mol % phosphatidylserine increased 4-fold faster than transfer to neutral vesicles, and transfer to vesicles containing 10 mol % cardiolipin was 4-fold faster. In contrast, transfer of retinol from CRBP-II was relatively unaffected by surface charge density, indicating that transfer from CRBP-II is not affected by the anionic character of the acceptor membranes. Taken together, the acceptor concentration and charge data suggest that these homologous binding proteins transfer retinol to model membranes via distinctly different mechanisms. CRBP-I may utilize collisional interactions with the membrane to effect retinol transfer, whereas retinol transfer from CRBP-II is likely to occur by aqueous diffusion of the dissociated ligand. The large enhancement of retinol transfer rate to acidic vesicles suggests that electrostatic interactions between CRBP-I and membranes may be involved in the retinol transfer mechanism.

Transfer of Retinaldehyde Versus Retinol from CRBPs to Membranes—CRBP-I and CRBP-II can bind both retinol and retinaldehyde with high affinity. The two proteins have equal affinity for retinaldehyde (11, 22), but CRBP-I appears to have a greater affinity for retinol than does CRBP-II (see above and Ref. 11). As both retinol and retinaldehyde serve as substrates for key metabolic enzymes, we compared the relative rates of transfer of these ligands to phospholipid membranes. Transfer of retinaldehyde from CRBP-II to EPC-SUVs was approximately 4-fold faster than transfer of retinol (on average 0.29 s⁻¹ for retinaldehyde and 0.07 s⁻¹ for retinol, Fig. 5A). Similarly, transfer of retinaldehyde from CRBP-I was 5-fold faster at the lowest lipid ratio examined (2.2 and 0.41 s⁻¹, respectively, Fig. 5B). Therefore, under these conditions both proteins transfer retinaldehyde to EPC membranes at a significantly faster rate than they transfer retinol. Increasing the concentration of acceptor membranes increased the rate of retinaldehyde transfer from CRBP-I but not from CRBP-II, indicating that for both proteins the mechanism of transfer of retinaldehyde is similar to that of retinol (Fig. 5).

Effect of Ionic Strength on Retinol Transfer—When diffusional movement through the aqueous phase is involved in the transfer of a hydrophobic ligand, increases in the ionic strength of the milieu can decrease its solubility and thereby decrease transfer rates logarithmically (26). If transfer occurs via effective collisions, such decreases in rate are not anticipated. Therefore, we examined the effects of ionic strength on transfer of retinol from the CRBPs by measuring the rate of transfer in the presence of increasing concentrations of NaCl (Fig. 6). Retinol transfer rates from CRBP-I increased significantly with an increase in ionic strength of the buffer. Approximately 85% of the total increase was reached by 0.5 m NaCl, and the
Transfer of retinol from holo-CRBP-I (1 μM) and CRBP-II (2 μM) was monitored to increasing concentrations of egg phosphatidylcholine vesicles as in Fig. 1. The average from three separate experiments ± S.D. is shown. **, p ≤ 0.03 and *, p ≤ 0.05 for retinol versus retinaldehyde.

Fig. 5. Retinol versus retinaldehyde transfer from CRBP's to phosphatidylcholine vesicles. The rate of transfer of ligand from 2 μM retinaldehyde-CRBP-II (●), 2 μM retinol-CRBP-II (●) (A), and 1 μM retinaldehyde-CRBP-I (●), 1 μM retinol-CRBP-I (●) (B) was monitored to increasing concentrations of egg phosphatidylcholine vesicles as in Fig. 1. The average from three separate experiments ± S.D. is shown. **, p ≤ 0.03 and *, p ≤ 0.05 for retinol versus retinaldehyde.

Fig. 6. Effect of ionic strength on retinol transfer from CRBPs. Transfer of retinol from holo-CRBP-I (1 μM, ■) or holo-CRBP-II (2 μM, ●) was measured as a function of NaCl concentration as described under “Experimental Procedures.” Acceptor phospholipid concentrations were 500 μM for CRBP-I and 200 μM for CRBP-II. Average rates from four experiments ± S.D. are shown.

Rate at 1 M NaCl was more than 3-fold greater than that at 0 M NaCl. These results are analogous to those obtained for the transfer of fatty acid from heart fatty acid-binding proteins to membranes, which is also postulated to occur by collisional interactions (25, 27, 28). Transfer rates from CRBP-II to membranes also increased in response to changes in ionic strength, although to a far lesser extent, with a difference of only 10% between 0 and 1 M NaCl (Fig. 6). Although these results for CRBP-II are not consistent with the expected decrease in transfer rate for an aqueous diffusion-mediated process, they nevertheless demonstrate substantial differences between the two CRBPs. Circular dichroism spectra showed that the molar ellipticity of the CRBPs did not change between 0.1 M NaCl and 0.5 M NaCl (data not shown), and therefore gross tertiary unfolding cannot account for the ionic strength effects seen in Fig. 6.

CRBP Interaction with Membranes Assessed Using Cytochrome c Binding Assays—Cytochrome c has been shown to interact with acidic membranes in vitro under conditions of low ionic strength (29, 30). We took advantage of this interaction by assessing whether preincubation with CRBPs modulates the fluorescence energy transfer between cytochrome c and acidic membranes containing 1 mol % of the fluorophore DPE as described under “Experimental Procedures.” Retinaldehyde was used as the ligand in these assays as it does not fluoresce under these spectroscopic conditions, whereas retinol does. As expected, cytochrome c addition resulted in a concentration-dependent quenching of the dansyl fluorescence (Fig. 7A). Fig. 7B demonstrates that preincubation of the vesicles with increasing amounts of CRBP-I-retinaldehyde was effective in preventing subsequent cytochrome c binding. Preincubation with 0.5 μM holo-CRBP-I resulted in a 48% increase in dansyl fluorescence over that seen in the absence of CRBP-I. In contrast, preincubation with CRBP-II had no effect on dansyl fluorescence. CRBP-I thus has a greater affinity for model membranes than does CRBP-II, in that it can prevent the binding of cytochrome c and the resultant quenching of dansyl fluorescence, whereas CRBP-II cannot protect against cytochrome c binding. These data are consistent with the results from the transfer assays, which suggested a membrane interaction-dependent ligand transfer mechanism for CRBP-I. Similar experiments with the apoproteins yielded somewhat inconsistent results (data not shown), perhaps reflective of subtle differences in the ability of apo and holo forms of the CRBPs to interact with membranes.

Adsortion of CRBP-I and CRBP-II to the Phospholipid/Water Interface—The interfacial adsorption rate and exclusion pressure for CRBP-I and CRBP-II were determined by injecting these proteins beneath EPC monolayers spread at increasing initial pressures (p) and determining the initial rate and final equilibrium change in surface pressure (ΔP). Following injection of the CRBPs into the buffer subphase, there was an immediate exponential increase in interfacial pressure (Fig. 8), indicating adsorption and insertion of CRBP-I molecules into the phospholipid monolayer. Rate constants for the initial adsorption of the CRBPs, calculated from the slope of the linear regression of time versus ln(ΔP), decreased with increasing initial surface pressure (Table I). At initial pressures up to and including 15 mN/m, the adsorption rates of CRBP-I were 2–3-fold faster than CRBP-II; at 20 mN/m the adsorption rates were similar. Equilibrium binding of CRBP-I to the phospholipid interface, as evidenced by the final ΔP, decreased with increasing p (Fig. 8, inset). Extrapolation of the p − ΔP curves to 0 indicated that CRBP-I could no longer penetrate the sur-
face at $p_i > 28.8$ mN/m, whereas CRBP-II could not penetrate the surface at $p_i > 25.9$ mN/m. These results indicate that CRBP-I interacts more strongly with the EPC monolayer than does CRBP-II. The absolute values of the exclusion pressures obtained here are lower than those similarly determined for the human plasma apolipoproteins, which are in the range of 29–34 mN/m (31–36), indicating that the CRBPs have lower intrinsic surface activity compared with the apolipoprotein family of lipid-binding proteins.

**DISCUSSION**

Several studies of the intracellular retinol-binding proteins have suggested that these proteins may play an important regulatory role in trafficking ligand to microsomal metabolic enzymes (5, 37). Integral to understanding the molecular basis of retinoid movement, therefore, is examining these potential interactions between binding proteins and membrane components. In this study we examined the ability of CRBP-I and CRBP-II to transfer retinol to phospholipid membranes as a means of modeling in vivo transfer to membranes and/or membrane-bound enzymes. By using changes in intrinsic tryptophan fluorescence as well as retinol fluorescence, we were able to determine the kinetics and mechanism of release of native ligand from the proteins to model membranes. The $\approx 6$-fold faster retinol transfer rates from CRBP-I compared with CRBP-II does not reflect the estimated 12-fold greater affinity of CRBP-I for this ligand. As $K_d$ is an equilibrium function reflecting the ratio of ligand dissociation and association rates, a discrepancy in magnitude between the relative equilibrium binding constants and the unidirectional ligand transfer rates may imply that retinol dissociation from the two proteins is occurring via different mechanisms and/or that retinol association rates are an order of magnitude higher for CRBP-II. The kinetic studies presented here indicate mechanistic differences in retinol transfer from CRBP-I versus CRBP-II to membranes. It is likely that the rates observed for ligand transfer from CRBP-II reflect spontaneous dissociation of ligand from the protein, whereas for CRBP-I the mechanism by which retinol leaves the protein appears to involve an interaction with the acceptor membrane. The fact that the rate of increase in tryptophan fluorescence upon retinol release from CRBP-II is identical to the rate of decrease of retinol fluorescence upon insertion into the NBD-labeled SUVs supports the interpretation of the transfer rates as a measure of ligand dissociation from the binding pocket.

The present studies show that transfer of retinol from...
CRBP-I and CRBP-II to membranes does occur. Moreover, it was found that transfer rates from CRBP-I are dependent on acceptor membrane concentration, implying that transfer may occur via protein-membrane “collisions”. These results are in contrast with two previously published studies. An earlier fluorometric study of retinol transfer rates from CRBP-I to model membranes found transfer rates from retinol CRBP-I to be independent of the acceptor vesicle concentration (9). Importantly, however, the lipid:protein ratios examined in that study ranged from 50:1 to 300:1. As those ratios do not exceed the retinol partition coefficient between CRBP-I and model membranes (determined here to be ~300:1), unidirectional transfer from protein to membranes was not being examined under those conditions, and evidence for a collisional mechanism would not be expected. A different study noted that the fluorescence spectrum of retinol bound to 1 nmol of CRBP-I did not change in the presence or absence of 25 µg of microsomal protein, suggesting that transfer of retinol to membranes from CRBP-I does not occur under those conditions (7). Assuming liver microsomes are composed of 30% lipid by weight (38) and using 800 as the average molecular weight of a phospholipid, the conditions employed in this latter study translate to a lipid:protein molar ratio of 9:1, far below the partition value and thus below the amount of membrane necessary to detect release of retinol from CRBP-I.

Retinol transfer from CRBP-II to membranes was independent of acceptor membrane concentration or charge, suggesting that transfer from CRBP-II occurs via diffusion of the ligand through the aqueous phase. Nevertheless, experiments examining the effects of ionic strength on transfer did not clearly support a diffusional transfer mechanism. Increases in the ionic strength of the solution would decrease the aqueous solubility of retinol, and a logarithmic decrease in transfer rate is expected when that transfer process involves aqueous diffusion. For CRBP-II, however, a small increase in rate was obtained with increasing salt. The explanation for this discrepancy is not known at this point. The changes may be due to slight alterations in the protein structure upon increasing NaCl (39) at levels undetectable by CD measurements, which are particularly insensitive to β-sheet structure. The substantial increase in retinol transfer rate with ionic strength for CRBP-I argues against a diffusion-mediated mechanism and may be a combination of protein structural effects and effects on the membrane surface. For example, increases in ionic strength can effect both bilayer structure and shield surface charges, two parameters likely to be important in the collisional mechanism proposed here (27). The large increase in transfer rate from CRBP-I is similar to ionic strength effects observed for the transfer of fatty acid from heart fatty acid-binding protein to phospholipid vesicles, which is likely to occur during collisional interactions between protein and membranes (25, 27, 28). Overall, these data demonstrate large differences between the two binding proteins in the modulation of retinol transfer by ionic strength and therefore support the differences observed for retinol transfer as a function of vesicle composition and concentration.

For both CRBPs, transfer of retinaldehyde to membranes occurred at a faster rate than transfer of retinol. Although it is difficult to correlate the transfer rates obtained in this model system with physiological processes in an absolute sense, the differences for CRBP-II may have an impact on retinoid flux through the intestine. In the enterocyte, CRBP-II obtains retinol from the hydrolysis of dietary retinyl esters or obtains retinaldehyde from cleavage of the more abundant β-carotene. CRBP-II-retinaldehyde undergoes reduction to retinol by a microsomal reductase. The CRBP-II-retinol obtained from dietary esters or via reduction is esterified by microsomal LRAT. In vitro determinations demonstrate that LRAT and the reductase have similar \( V_{\text{max}} \) values in intestinal mucosal microsomes (40). The data in Fig. 5A suggest that within the cell the reductase may obtain its retinaldehyde substrate from CRBP-II at a greater rate than LRAT obtains its retinol substrate and that despite similar maximal velocities, net flux through the reductase may therefore be greater. As the product of the reductase reaction must next be esterified by LRAT, the results suggest that in the enterocyte the esterification by LRAT, and not the reduction by reductase, may be rate-determining in processing β-carotene-derived vitamin A.

In addition to the kinetic evidence suggesting that CRBP-I is able to interact effectively with lipid vesicles during the process of retinol transfer, the protection against cytochrome \( c \) binding directly demonstrates that CRBP-I is membrane-interactive. In contrast, CRBP-II is not able to prevent the subsequent binding of cytochrome \( c \), indicating that it cannot interact with membranes as effectively as CRBP-I. These observations are supported by the monolayer exclusion data that demonstrate that CRBP-I is more surface-active than CRBP-II. Both the rate of adsorption and interfacial exclusion pressure are greater for CRBP-I than for CRBP-II, which may provide an explanation for the increased rate of transfer from CRBP-I, as the association of CRBP-I with membranes is both faster and stronger than the association of CRBP-II. For CRBP-II it appears that the degree of membrane interaction is insufficient to obtain regulation of the ligand transfer rate by membrane properties, a hallmark of collisional ligand transfer.

Results from these model systems cannot only be extended to cellular lipid components but may also extend to the mechanism of transfer from these proteins to charged protein components, e.g. enzymes, within cellular membranes. The data indicate that the molecular interaction of CRBP-I and CRBP-II with cellular membranes and membrane-bound enzymes may be markedly different. Transfer of retinol to membrane enzymes could involve direct protein interaction with the membrane, direct interaction with the enzyme, or a combination of both. The fact that holo-CRBP-II can present ligand to microsomal enzymes (7) but does not effectively interact with model membranes in ligand transfer suggests that interactions between CRBP-II and membrane-bound enzymes may be governed by direct protein-protein interactions. In contrast, the evidence here for collisional transfer of retinoids from CRBP-I suggests that interaction between CRBP-I and membrane enzymes could include recognition of a lipid component, allowing for specific and selective discernment of the binding protein by metabolic enzymes in particular membrane environments.

The cellular retinol-binding proteins are members of the intracellular fatty acid-binding superfamily. The structure of these proteins consists of 10 strands of anti-parallel β-sheet capped by two short α-helices. This family of binding proteins displays flexibility in the helical cap region, and it has been hypothesized that these helices represent a portal cap for the ligand binding pocket (41–47). This laboratory has demonstrated that distinct members of the fatty acid-binding protein (FABP) family can transfer fatty acid to model membranes via direct interactions with membranes (reviewed in Ref. 48). Specifically, the helix-turn-helix domain of intestinal FABP was shown to be critical for the collisional transfer of fatty acid to membranes, and mutagenesis studies have implicated the amphipathic α-helix-I of heart-type FABP as important in dictating electrostatic interactions with membranes (20, 25). Amphipathic helices are known to be involved in protein-membrane interaction via their ability to orient proteins at the polar/nonpolar interface of a membrane (49, 50). Crystallo-
are related to the number and amphipathicity of hydrophobic residues. Transfer from CRBP-II, on the other hand, may not occur primarily via a diffusional mechanism and may not involve a lipid component. These differences may underlie, at least in part, the distinct functional roles of these proteins in the modulation of intracellular retinoid metabolism.

REFERENCES

1. Ho, M. T. P., Pownall, H. J., and Hollyfield, J. G. (1989) J. Biol. Chem. 264, 17759–17763
2. Noy, N., and Xu, X. J. (1990) Biochemistry 29, 3878–3883
3. Fex, G., and Johannesson, G. (1987) Biochim. Biophys. Acta 901, 255–264
4. Sundaram, M., Sivaprasadara, A., DeSousa, M. M., and Findlay, J. B. C. (1997) J. Biol. Chem. 272, 3336–3342
5. Ong, D. E., Newcomer, M. E., and Chytil, F. (1994) in The Retinoids: Biology Chemistry and Medicine (Sporn, M. B., Roberts, A. B., Goodman, D. S., eds) pp. 283–317, Raven Press, Ltd., New York
6. Li, E., and Norris, A. W. (1996) Annu. Rev. Nutr. 16, 205–234
7. Herr, F. M., and Ong, D. E. (1993) Biochemistry 31, 6748–6755
8. Napoli, J. L. (1993) J. Nutr. 123, 362–366
9. Noy, N., and Blaner, W. S. (1991) Biochemistry 30, 6380–6386
10. Rong, D., Lin, C.-L. S., d'Avignon, A., Lovey, A. J., Rosenberger, M., and Li, E. (1997) FEBS Lett. 402, 116–120
11. Li, E., Qian, S., Winter, N., d'Avignon, A., Levin, M., and Gordon, J. J. (1991) J. Biol. Chem. 266, 3622–3629
12. Storch, J., and Kleinfeld, M. A. (1986) Biochemistry 25, 1717–1726
13. Gomori, G. (1942) J. Lab. Clin. Med. 27, 955–960
14. Cogan, U., Kopleman, M., Mokady., S., and Shinitsky, M. (1976) Eur. J. Biochem. 65, 71–78
15. Massey, J. B., Bick, D. H., and Pownall, H. J. (1997) Biochem. J. 328, 1723–1743
16. Nichols, J. W., and Pagano, R. E. (1982) Biochemistry 21, 1720–1726
17. Hsu, K.-T., and Storch, J. (1990) J. Biol. Chem. 265, 13317–13323
18. Faucon, J. P., Dufourcq, J., Lassan, C., and Bernon, H. (1976) Biochim. Biophys. Acta 415, 283–294
19. Mustonen, P., Vistanen, J. A., Somerharju, P., and Kinnenun, P. K. J. (1987) Biochemistry 26, 2991–2997
20. Corsi, B., Cistola, D. P., Frieden, C., and Storch, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 12174–12178
21. Weinberg, R. B., Cook, V. R., Jones, J. B., Kussie, P., and Tall, A. R. (1994) J. Biol. Chem. 269, 29588–29591
22. MacDonald, P. N., and Ong, D. E. (1987) J. Biol. Chem. 262, 10550–10556
23. Levin, M. S., Li, E., Ong, D. E., and Gordon, J. J. (1987) J. Biol. Chem. 262, 7118–7124
24. Herr, F. M., Matarese, V., Bernlohr, D. A., and Storch, J. (1995) Biochemistry 34, 11840–11845
25. Herr, F. M., Aronson, J., and Storch, J. (1996) Biochemistry 35, 1296–1303
26. Kim, H. K., and Storch, J. (1992) J. Biol. Chem. 267, 77–82
27. Kim, H. K., and Storch, J. (1992) J. Biol. Chem. 267, 20051–20056
28. Wootan, M. G., and Storch, J. (1994) J. Biol. Chem. 269, 16151–16153
29. Ross, D., and Storch, J. (1995) J. Biol. Chem. 270, 3197–3199
30. Krebs, E. E., Ibah, J. A., and Phillips, M. C. (1988) Biochemistry Biophys. Acta 959, 229–237
31. Krebs, E. E., Phillips, M. C., and Sparks, C. E. (1983) Biochim. Biophys. Acta 751, 470–475
32. Ibah, J. A., and Phillips, M. C. (1988) Biochemistry 27, 7155–7162
33. Ibah, J. A., Krebs, E. E., and Phillips, M. C. (1989) Biochim. Biophys. Acta 1004, 300–308
34. Ibah, J. A., Lund-Katz, S., and Phillips, M. C. (1989) Biochemistry 28, 1126–1133
35. Weinberg, R. B., Ibah, J. A., and Phillips, M. C. (1992) J. Biol. Chem. 267, 8977–8983
36. Napoli, J. L., Posch, K. P., Fiorella, P. D., and Boerman, M. H. E. M. (1991) Biochemical Pharmacother. 45, 131–143
37. Depierre, J. W., and Dallner, G. (1975) Biochim. Biophys. Acta 415, 411–472
38. Yun, C.-H., Song, M., Ahn, T., and Kim, H. (1996) J. Biol. Chem. 271, 31312–31316
39. Herr, F. M., Wardlaw, S. A., Kakkad, B., Albrecht, A., Quick, T. C., and Ong, D. E. (1993) J. Lipid Res. 34, 1545–1554
40. Winter, N. S., Bratt, M. J., and Banaszak, L. J. (1993) J. Mol. Biol. 230, 1247–1259
41. Cowan, S. W., Newcomer, M. E., and Jones, T. A. (1993) J. Mol. Biol. 230, 1225–1246
42. Rizzi, J., Liu, Z.-P., and Gierasch, L. M. (1994) J. Biomol. NMR 4, 741–760
43. van Aalten, D. M. F., Findlay, J. B. C., Amandel, A., and Berendsen, H. J. C. (1995) Protein Eng. 8, 1129–1135
44. van Aalten, D. M. F., Jones, P. C., de Sousa, M., and Findlay, J. B. C. (1997) Protein Eng. 10, 31–37
45. Hodsdon, M. E., and Cistola, D. P. (1996) Biochemistry 35, 1450–1460
46. Hodsdon, M. E., and Cistola, D. P. (1997) Biochemistry 36, 2278–2290
47. Storch, J., Herr, F. M., Hsu, K.-T., Kim, H. K., Liou, L. I., and Smith, E. F. (1999) Comp. Biochem. Physiol. 115, 333–339
48. Anantharamaiah, G. M., Jones, M. K., and Segrest, J. P. (1993) in The Amphipathic Helix (Expand, R. M., ed) pp. 110–143, CRC Press, Inc., Boca Raton, FL
49. Roise, D. (1993) in The Amphipathic Helix (Expand, R. M., ed) pp. 258–283, CRC Press, Inc., Boca Raton, FL