Distinct Roles for Cellular Retinoic Acid-binding Proteins I and II in Regulating Signaling by Retinoic Acid*

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The pleiotropic effects of retinoic acid (RA) in mammalian cells are mediated by two classes of proteins: the retinoic acid receptors (RAR) and cellular retinoic acid-binding proteins (CRABP-I and CRABP-II). Here we show that expression of CRABP-II, but not CRABP-I, markedly enhanced RAR-mediated transcriptional activation of a reporter gene in COS-7 cells. The equilibrium dissociation constants of complexes of CRABP-I or CRABP-II with RA were found to differ by 2-fold. It is thus unlikely that the distinct effects of the two proteins on transcription stem from differential ligand-binding affinities. The mechanisms by which RA transfers from the CRABPs to RAR were thus investigated directly. The rate constant for movement of RA from CRABP-II, but not from CRABP-I, to RAR strongly depended on the concentration of the acceptor. The data suggest that transfer of RA from CRABP-I to RAR involves dissociation of the ligand from the binding protein, followed by association with the receptor. In contrast, movement of RA from CRABP-II to the receptor is facilitated by a mechanism that involves direct interactions between CRABP-II and RAR. These findings reveal a striking functional difference between CRABP-I and CRABP-II, and point at a novel mechanism by which the transcriptional activity of RA can be regulated by CRABP-II.

*This work was supported by National Institutes of Health Grant CA68150 and Grant 5-T32-DK07158 (to D. J. L.) and by United States Department of Agriculture Grant 89-34115-4498. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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PROTEINS—

RA with a high affinity comprises two homologous proteins, cellular RA-binding proteins I and II (CRABP-I and CRABP-II). CRABPs are found in all vertebrates and are highly conserved across species (2). The two CRABP isoforms display different patterns of expression across cells and developmental stages. In the adult, CRABP-I is expressed almost ubiquitously, whereas CRABP-II is only expressed in skin (2), uterus, ovary (3, 4), and in the choroid plexus (5). Both CRABPs are widely expressed in the embryo, although they do not usually co-exist in the same cells (6). The distinct patterns of expression of CRABP-I and II suggest that they serve different functions in the biology of RA, or, perhaps, that they allow for accommodating different requirements for RA in different tissues. Although the genes for both CRABP isoforms from various species have been cloned and characterized, neither the exact functions of these proteins nor the distinct roles of the two isoforms are completely understood at present. Interestingly, despite the high conservation of CRABPs, mice in which these genes have been disrupted appear essentially normal (7). It is usually proposed that CRABPs serve to solubilize and protect their ligand in cytosol and that they transport RA between different cellular compartments (2). It was suggested, for example, that CRABPs act to deliver their ligand to the nucleus (8). Indeed, it was recently demonstrated that both CRABP isoforms are present not only in cytosol but also in the nuclei of cells (9).

It was also suggested that CRABP-I regulates the metabolic fate of its ligand by directly affecting the activities of RA-metabolizing enzymes. It was reported that the rate of degradation of RA in F9 teratocarcinoma cells increases upon elevation of the expression level of CRABP-I (10). It was shown further that the sensitivity of F9 cells to RA-induced differentiation is inversely correlated to the cellular level of CRABP-I (11). Hence, it is currently believed that CRABP-I modulates cellular response to RA by facilitating catabolism and/or by sequestering RA, rendering it unavailable to nuclear receptors.

Little information is available regarding the specific biological role of CRABP-II. Here, the kinetic patterns that govern the process by which CRABPs deliver their ligand to RAR were examined and correlated with their effects on RAR-mediated transcriptional activation. The observations indicate that CRABP-II, but not CRABP-I, directly interacts with RAR and that these protein-protein interactions markedly facilitate the formation of the RAR-RA complex.

EXPERIMENTAL PROCEDURES

Proteins—RARs Lacking the terminal A/B domain (RARsΔAB) was obtained by overexpression in Escherichia coli and purified as described previously (13). This protein displays ligand-binding, DNA-binding, and dimerization properties that are identical to those of the full-length protein (14). Bacterial expression vectors for bCRABP-I and bCRABP-II (in pT7 vector) were provided by David Ong (Vanderbilt University). These proteins were expressed and purified as described (15).

Transactivation Assays—COS-7 cells were transfected with pSG5 lacking the terminal A/B domain (RARsΔAB) was obtained by overexpression in Escherichia coli and purified as described previously (13). This protein displays ligand-binding, DNA-binding, and dimerization properties that are identical to those of the full-length protein (14). Bacterial expression vectors for bCRABP-I and bCRABP-II (in pT7 vector) were provided by David Ong (Vanderbilt University). These proteins were expressed and purified as described (15).

Fluorescence Titrations—were carried out as described previously (16) and followed either by monitoring the ligand-induced decrease of the fluorescence of the protein (λex = 280 nm, λem = 340 nm) or the increase in fluorescence of RA upon binding to CRABP (λex = 360 nm, λem = 470 nm) (18). Titration curves were corrected (24) and data fitted to a binding equation (19).
CRABP-I and II in RA Signaling

CRABP-I and II Deliver RA to RAR by Different Mechanisms—It is difficult to see how a 2-fold difference in the $K_d$ of CRABP-I and II may account for their distinct effects on the transcriptional activity of RAR. We thus wondered whether the functional differences between the two proteins may stem from differences in the mechanisms by which they deliver their ligand to RAR. Theoretically, transfer of a ligand from a donor to an acceptor protein may occur by one of two possible mechanisms. One pathway involves initial dissociation of the ligand from the donor into the aqueous phase, followed by association with the acceptor. In this case, the rate-limiting step for the
dissociation and association of complexes of RA with CRABP-I or CRABP-II. Rates of dissociation of RA from CRABP-I (a) or CRABP-II (b) were measured as described under “Experimental Procedures.” Holo-proteins were mixed with vesicles of DOPC at a molar ratio of 20,000. Transfer was followed by monitoring the time-dependent decrease in the fluorescence of RA. Data were fitted to a first order reaction equation to yield $k_{off}$. Rates of association of RA with CRABP-I (c) or CRABP-II (d) were monitored following mixing solutions containing equimolar concentrations of the binding protein and RA (final concentration 0.5 μM each). Mixing was carried out using a stopped-flow apparatus in conjunction with the fluorometer. The fluorescence of RA was monitored, and the rate constant for association ($k_{on}$) was obtained by fitting the data to a second order reaction equation.

RESULTS

CRABP-II, but Not CRABP-I, Enhances Transcriptional Activation by RA—To investigate whether either CRABP-I or CRABP-II affects the transcriptional activity of RA, transactivation assays were carried out. A CAT reporter construct containing the response element DR-5, which specifically binds RAR-RXR heterodimers (21), was co-transfected into COS-7 cells together with expression vectors for either CRABP-I or CRABP-II, and the ability of RA to activate transcription of the reporter gene was studied (Fig. 1). Addition of RA induced expression of the reporter in a dose-dependent fashion (Fig. 1, open bars). Overexpression of CRABP-I had little effect on the RA-induced activation (Fig. 1, gray bars). In contrast, expression of CRABP-II markedly stimulated transactivation by RA (Fig. 1, hatched bars) in this system.

The Equilibrium Dissociation Constants of Complexes of CRABP-I and CRABP-II with RA Are Similar—To examine whether functional differences between CRABP-I and CRABP-II may stem from differences in their RA binding affinity (19), $K_d$ values characterizing the association of RA with CRABP-I and CRABP-II were measured. To this end, the kinetic parameters of the dissociation and the association of complexes of RA with the binding proteins ($k_{off}$ and $k_{on}$, respectively) were measured. The respective $K_d$ values were obtained by using the relationship $K_d = k_{off}/k_{on}$.

To withdraw RA from CRABP, unilamellar vesicles of DOPC, serving as a “hydrophobic sink,” were used (22). CRABP was pre-complexed with RA, mixed with the vesicles, and movement of the ligand from the protein to the vesicles was followed. RA fluoresces when bound to CRABP-I or II, although it is not fluorescent when associated with lipid vesicles (18). RA transfer from CRABP to vesicles could thus be followed by monitoring the time-dependent decrease in the fluorescence of the ligand. In this assay, because the amount of vesicles used was sufficient to draw >95% of the ligand from the protein, the rate constant of the observed reaction directly reflects the rate constant for dissociation of RA from the protein ($k_{off}$) (16, 20).

Representative traces showing transfer of RA from CRABP-I or CRABP-II to vesicles are shown in Fig. 2, a and b, respectively, and the derived rate constants are listed in Table I. The rate of association of the CRABP-RA complexes was examined by mixing equimolar concentrations of RA and protein and following the time-dependent enhancement of the fluorescence of the ligand upon binding (Fig. 2, c and d). These data were analyzed as described previously (20) to yield $k_{on}$ (Table I). The data indicated that $k_{on}$ for the two proteins are similar and close to the diffusion limit and that $k_{off}$ is 2-fold lower for CRABP-I versus CRABP-II, leading to a 2-fold higher ligand-binding affinity of the former.

CRABP-I and CRABP-II Deliver RA to RAR by Different Mechanisms—It is difficult to see how a 2-fold difference in the $K_d$ of CRABP-I and II may account for their distinct effects on the transcriptional activity of RAR. We thus wondered whether the functional differences between the two proteins may stem from differences in the mechanisms by which they deliver their ligand to RAR. Theoretically, transfer of a ligand from a donor to an acceptor protein may occur by one of two possible mechanisms. One pathway involves initial dissociation of the ligand from the donor into the aqueous phase, followed by association with the acceptor. In this case, the rate-limiting step for the
transfer reaction will be the dissociation of the donor-ligand complex, and the rate constant of the reaction will be independent of the nature or the concentration of the acceptor (23). In a second scenario, the ligand will move from the donor to the acceptor by “channeling,” i.e. by a process that involves direct protein-protein interactions and that bypasses the aqueous phase. In this case, the rate of the reaction will be limited by the frequency of productive collisions between the donor and the acceptor and will become faster as the acceptor/donor ratio is increased.

Hence, the mechanism by which CRABP “delivers” RA to RAR can be delineated by examining the dependence of the rate constants of ligand transfer between the two proteins on the concentration of the acceptor (RAR). As RA is an efficient fluorophore when bound to either CRABP but not when associated with RAR, transfer could be followed by the time-dependent decrease in RA fluorescence upon mixing of holo-CRABP with apo-RAR. The rate constant for movement of the RA from CRABP-I to RAR was independent of the concentration of the acceptor (Fig. 3a), indicating that movement of RA from CRABP-I to RAR requires prior dissociation of the ligand from CRABP-I. To verify the accuracy of the fluorescence measurements, the rate of transfer was also measured by monitoring movement of $^3$H-RA from CRABP-I to RAR. Following mixing, separation of the two proteins at different time points was affected by addition of Ni$^{2+}$ chelating beads which bind the his-tagged RAR but not CRABP. Mixtures were centrifuged, and the remaining CRABP-bound RA was measured by counting. The rate constant thus obtained was essentially identical to that extracted from the fluorescence assays (data not shown).

In contrast with CRABP-I, the pseudo first order rate constant for movement of RA from CRABP-II to RAR (Fig. 3b) strongly depended on the concentration of the acceptor; a 5-fold increase in the concentration of RAR facilitated that rate of transfer of RA from CRABP-II to RAR by 5-fold. This behavior strongly indicates that movement of RA from CRABP-II to RAR is mediated by direct protein-protein interactions between the two proteins.

Overall, the data reveal that while CRABP-I acts as a passive vehicle for RA which binds and releases its ligand in response to shifts in equilibrium conditions, CRABP-II delivers RA to RAR by “channeling” between the two proteins. These observations show further that the interactions between CRABP-II and RAR result in significant facilitation of the formation of the RAR-RA complex and may be the basis for the enhancing effect of CRABP-II on the RAR-mediated transactivation.

**The Interactions between CRABP-II and RAR Are Transient**—To further characterize the interactions that mediate transfer of RA between CRABP-II and RAR, we attempted to demonstrate formation of a stable complex between the two proteins. The following methods were used: 1) chemical cross-linking (13), 2) electrophoresis under nondenaturing conditions, 3) fluorescence anisotropy titrations (24, 25), 4) electrophoretic mobility-shift assays, intended to examine whether cognate DNA may stabilize the complex (25). We could not detect complex formation using any of these methods. It thus seems that the interactions of CRABP-II-RAR complex is an unstable intermediate with a short half-life.

**DISCUSSION**

Two classes of proteins are believed to be involved in regulating the transcriptional activities of RA: the retinoid nuclear receptors RAR and RXR, and the cellular RA-binding proteins CRABP-I and CRABP-II. The mechanisms of action of retinoid receptors have become increasingly understood in recent years. However, despite the striking level of conservation of CRABP across species, which suggest that they play critical roles in RA action, and although the remarkably different expression profiles of the two CRABP isoforms suggest that they play different roles in RA biology, neither the exact functions of these proteins nor the nature of their distinct roles are clear at present. The current study was thus undertaken to investigate possible functional differences between CRABP-I and CRABP-II.

The data presented in Fig. 1 demonstrate that expression of CRABP-II markedly stimulates the RA-induced transcriptional...
activity of RAR and that, in contrast, CRABP-I has no effect on this activity. Hence, the two proteins display remarkable functional differences.

It was previously shown that elevated expression of CRABP-I in F9 teratocarcinoma cells inhibits the transcriptional activity of RAR, and it was suggested that CRABP-I directs RA to enzymes that catalyze its degradation (10, 11). In contrast, the data in Fig. 1, in agreement with previous studies carried out using CV-1 cells (26), show that expression of CRABP-I had little effect on RA-induced transactivation in COS-7 cells. It thus seems that the effect of CRABP-I on the transcriptional activity of RAR depends on the particular cell type. It is reasonable to suggest, for example, that CRABP-I inhibits the transcriptional activity of RA only in cells that express RA-metabolizing enzymes that are under its direct regulation. Such enzymes were demonstrated to exist in F9 cells (11). To our knowledge, metabolism of RA in either COS-7 or CV-1 has not been studied in depth.

The $K_d$ values of complexes of RA with CRABP-I and -II were found to differ by only 2-fold (Table I), suggesting that it is unlikely that the functional differences between the two proteins stem from differential ligand-binding affinities. We thus set out to examine whether CRABP-I and -II might deliver their ligand to RAR by different mechanisms. Theoretically, there are two possible pathways by which a ligand can move from a donor to an acceptor protein. Transfer may proceed by dissociation of the ligand from the donor to the aqueous phase, followed by association with the acceptor. In this scheme, the rate of transfer will be limited by the rate of dissociation of the donor-ligand complex. Alternatively, the ligand may transfer from the donor to the acceptor by a process that is mediated by direct protein-protein interactions. In this case, the rate of transfer will depend on the probability of productive collisions between the two proteins and will be faster as the acceptor/donor ratio is increased. Studies of the kinetic patterns of the movement of RA between the binding proteins and RAR (Fig. 3) demonstrated that transfer of RA from CRABP-I to RAR is indicative of a process that requires prior dissociation of the RA-CRABP-I complex (see Fig. 4a). This conclusion is also supported by the observations that the rate constant for dissociation of RA from CRABP-I was similar regardless of whether the acceptor was RAR or lipid vesicles (Table I).

In contrast with CRABP-I, the pattern of movement of RA from CRABP-II to RAR was characteristic of a process that is mediated by direct interactions between the two proteins (see Fig. 4b). Further, the data showed that these interactions result in a significant facilitation of the delivery of RA to the receptor. Hence, CRABP-II enhances the transcriptional activity of RAR by directly interacting with the receptor, thereby facilitating the formation of the active RAR-RAR complex. Interestingly, it was recently reported that ectopic expression of CRABP-II enhances transcriptional activation by RA in mammary carcinoma cell lines (27) and that expression of a CRABP-II antisense construct in SCC25 cells renders these cells less sensitive to RA-mediated inhibition of proliferation (12). No suggestions for the mechanism that may underlie these observations were put forward in these reports, but the results of the present work suggest that these effects were mediated by the CRABP-II-dependent facilitation of the formation of holo-RAR. It is also worth noting that it has been reported that the expression of CRABP-II is elevated in cells that synthesize relatively large amounts of RA (5, 28, 29). These observations can be understood, in view of the present findings, to imply that increased physiological needs for RA require both an increase in RA synthesis and an up-regulation of CRABP-II, allowing for rapid delivery of newly synthesized RA to RAR.

Our efforts to demonstrate a stable complex between CRABP-II and RAR failed despite utilization of multiple experimental approaches. It thus seems that the interactions between the two proteins are transient in nature and that the CRABP-II-RAR complex is a short-lived intermediate. Transient interactions that serve to channel ligands between proteins have been previously documented for several enzymes, for example within the purine biosynthetic pathway (30). In these cases, similar to the present study, it was not possible to isolate a stable complex between the proteins involved although it has been established that direct protein-protein interactions indeed occur and play an important role in this particular metabolic pathway. To the best of our knowledge, the present work constitutes the first example for channeling of a small ligand between two proteins for purposes other than movement of a substrate down a metabolic pathway.

Acknowledgments—We thank Anuradha Budhu for measurements of rates of transfer of $^{3}H$-RA between CRABP-I and RAR. We are grateful to David Ong, for providing bacterial expression vectors for CRABP-I and CRABP-II, and to Hinrich Gronemeyer and Pierre Chambon, for cDNA for CRABP-I, CRABP-II, and RAR and for the DR5-tk-CAT reporter construct.

REFERENCES

1. Chambon, P. (1996) FASEB J. 10, 940–954
2. Ong, D. E., Newcomer, M. E. & Chytil, F. (1994) in Retinoids: Biology, Chemistry and Medicine (Sporn, M. B., Roberts, A. B. & Goodman, D. S., eds) 2nd Ed., pp. 288–317, Raven Press, NY
3. Zheng, W. L. & Ong, D. E. (1998) Biol. Reprod. 58, 963–970
4. Wardlaw, S., Bucco, R. A., Zheng, W. L. & Ong, D. E. (1997) Biol. Reprod. 56, 125–132
5. Yamamoto, M., Drager, U. C., Ong, D. E. & McCaffery, P. (1998) Eur. J. Biochem. 257, 344–350
6. Maden, M. (1994) in Vitamin A in Health and Disease (Blomhoff, R., ed) pp. 289–322, Marcel Dekker, NY
7. Gorry, P., Luflin, T., Dierich, A., Rochette-Egly, C., Decimo, D., Delle, P., Mark, M., Durand, B. & Chambon, P. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9032–9036
8. Takase, S., Ong, D. E. & Chytil, F. (1986) Arch. Biochem. Biophys. 247, 328–334
9. Gaub, M.-P., Lutz, Y., Ghyseleinck, N. B., Scheuer, I., Pfister, V., Chambon, P., & Rochette-Egly, C. (1988) J. Histochem. Cytochem. 46, 1103–1111
10. Boylan, J. F. & Gudas, L. J. (1992) J. Biol. Chem. 267, 21486–21491
11. Boylan, J. F. & Gudas, L. J. (1991) J. Cell Biol. 112, 965–975
12. Vs. H. P. & Crowe, D. L. (1998) Anticancer Res. 18, 217–224
13. Kersten, S., Kelleher, D., Chambon, P., Gronemeyer, H. & Noy, N. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10014–10017
14. Chen, Z.-P., Iyer, J., Bourguet, W., Held, P., Mioskowski, C., Lebeau, L., Noy, N., Chambon, P. & Gronemeyer, H. (1996) J. Mol. Biol. 275, 55–65
15. Jamison, R. S., Newcomer, M. E. & Ong, D. E. (1994) Biochemistry 33, 2873–2879
16. Kersten, S., Dawson, M. L., Lewis, B. A. & Noy, N. (1996) Biochemistry 35, 3816–3824
17. Cogan, U., Kopelman, M., Mokady, S. & Shinitzky, M. (1976) Eur. J. Biochem. 65, 71–78
18. Fiorella, P. D., Giguere, V. & Napoli, J. L. (1993) J. Biol. Chem. 268, 21545–21552
19. Norris, A. W., Cheng, L., Giguere, V., Rosenberger, M. & Li, E. (1994) Biochim. Biophys. Acta 1209, 10–15
20. Nay, N. & Xu, Z.-J. (1990) Biochemistry 29, 3878–3883
21. Mangelsdorf, D. J., Umesono, K. & Evans, M. R. (1994) in The Retinoids: Biology, Chemistry and Medicine (Sporn, M. B., Roberts, A. B. & Goodman, D. S., eds) 2nd Ed., pp. 319–349, Raven Press, NY
22. Chen, Y., Houghton, L. A., Brenna, J. T. & Noy, N. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10691–10700
23. Kon, R. & Chambon, P. (1997) Nucleic Acids Res. 25, 20507–20515
24. Daniels, C., Nay, N. & Zakim, D. (1985) Biochemistry 24, 3288–3292
25. Lakowicz, J. R. (1983) in Principles of Fluorescence Spectroscopy, pp. 112–150, Plenum Press, New York
26. Dong, D. & Noy, N. (1998) Biochemistry 37, 10691–10700
27. Venema, P., Reddy, L. G. & Sani, B. P. (1996) Biochemistry 35, 9974–9982
28. Jamison, R. S., Newcomer, M. E. & Ong, D. E. (1994) Biochemistry 33, 20507–20515
29. Fiorella, P. D., Giguere, V. & Napoli, J. L. (1993) J. Biol. Chem. 268, 21545–21552
30. Rudolph, J. & Stabbe, J. (1995) Biochemistry 34, 2241–2245