STUDIES ON THE MECHANISMS OF LIGAND-INDUCED NUCLEAR LOCALIZATION OF CELLULAR RETINOIC ACID BINDING PROTEIN TYPE II

A Thesis

Presented to the Faculty of the Graduate School

Of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

Richard J. Sessler

January 2006

ABSTRACT

Retinoic acid (RA), a derivative of vitamin A, is well know for its ability to act as a transcriptional modulator, and it is critically involved in a number of cellular processes. The cellular retinoic acid binding proteins (CRABP-I and II) are small cytosolic proteins that bind to RA with a high affinity, and they are thought to play a role in the biological activities of RA. The expression of CRABP-II has been shown to selectively enhance the activity of a group of ligand-inducible transcription factors known as the retinoic acid receptors (RARs), and CRABP-II has also been shown to accumulate in the nucleus in response to RA treatment. CRABP-II is thought to enhance RAR activity by enhancing the movement of RA into the nucleus, where RAR functions as a transcription factor. The question addressed in this work was that of the mechanism by which CRABP-II accumulates in the nucleus in response to RA.

Abolishing nuclear localization of CRABP-II, and the resulting effects on RAR activity were addressed. CRABP-II was tagged to a nuclear export signal (NES), which actively excludes a protein from the nucleus. Subsequently, the NES functionality was verified by fluorescence microscopy. Functional assays for RAR activity were performed, and it was determined that accumulation of CRABP-II in the nucleus was necessary for it to enhance the activity of RAR.

To address possible mechanisms for the nuclear localization of CRABP-II, analysis of its primary and tertiary structures were performed. No nuclear localization signals could be identified in the primary sequence of CRABP-II. Comparisons of the electrostatic surface potentials of apo- and holo- crystal structures of CRABP-II identified a region of the protein that appeared to become more basic in response to RA binding. Three basic

residues in the region were identified as potentially important for the change in the surface potential. Further analysis identified the residues as being very similar in orientation to a classical primary nuclear localization signal.

The three basic residues of CRABP-II were converted to alanines by mutagenesis, and the recombinant mutant was purified from *E. Coli*. Using the purified WT and mutant proteins, it was determined that mutation of the residues did not significantly change the proteins' sensitivity to urea-induced denaturation, and that the mutations did not significantly disturb the ability of CRABP-II to bind RA.

The three basic residues were then mutated in mammalian expression vectors, and the effects of mutating them were evaluated by microscopy. It was determined that the three basic residues are required for RA induced nuclear localization of CRABP-II. Further, it was determined that Leptomycin-B (LMB), an inhibitor of nuclear export, had no effect on the RA-mediated localization of the WT or the mutant protein, suggesting that CRABP-II nuclear localization is mediated by an import machinery.

Functional assays for RAR activity were used to address the functional significance of the three basic residues. It was determined that the mutant protein was unable to enhance the activity or RAR to the same degree as WT, and further that the enhancement by the mutant was similar to that obtained using the NES-tagged WT protein.

Further research was done to identify the proteins involved with the RA induced nuclear import of CRABP-II. Recombinant CRABP-II-GST was shown to interact with importinα, a known nuclear import-related protein, in an RA dependent fashion. The NLS CRABP-II mutant did not interact with importinα

even in the presence of RA, further supporting the hypothesis that RA induced nuclear localization requires the three NLS residues.

Overall, this work suggests a unique mechanism for nuclear import, in which a non-classical NLS emerges in CRABP-II in response to RA binding.

BIOGRAPHICAL SKETCH

Richard Sessler was born in Binghamton, NY on March 4th, 1973. He began his academic career at Broome Community College in Binghamton in 1997, and completed an A.A. there in 1999. He went on to complete a B.S. in biology at Binghamton University in 2001. After his bachelors was completed, he moved to Ithaca NY, and in 2001 he joined the lab of Dr. Noa Noy at Cornell University as a laboratory technician. After a year as a technician, he joined the Ph.D. program in nutritional sciences and continued his work in the Noy lab. He currently resides in Columbus, Ohio.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Noa Noy, for her support, and for her unique introduction to modern laboratory research.

I am very grateful to Dr. Noa Noy (grant # R01-CA68150) and to the National Institutes of Health (grant # T32-CA09682) for providing financial support during my training.

Thank you also to my special committee members, Dr. Rick Cerione, Dr. David Shalloway, and Dr. Paul Soloway for your time and support.

I would also like to thank the Cornell Division of Nutritional Sciences for providing me with a small grant for research supplies that were used in this work.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 RETINOIC ACID

Retinoic acid (RA) is a labile, 20 carbon fatty acid consisting of a β-ionone ring attached to an isoprenoid chain which ends with a carboxylic acid moiety (figure 1.1). RA is a derivative of vitamin A, an essential nutrient that cannot be synthesized endogenously by mammals. Vitamin A is taken up from the diet, either from the pro-vitamin A carotenoids found in plants, or from retinyl esters found in animal products. The circulating form of vitamin A, retinol (ROH) can be converted into RA by a number of different tissues and cell types, including the uterus (Bucco et al., 1997), and the testes (Cavazzini et al., 2003). It is generally believed that retinoic acid is synthesized from ROH in cells by a two-step process in which ROH is first reversibly converted into retinal (RAL), and RAL is subsequently converted irreversibly into RA (Napoli, 1999). The conversion of ROH into RAL proceeds via a number of enzymes that are members of the short-chain alchohol dehydrogenase/reductase (SDR) family as well as the alcohol dehydrogenase (ADH) family. At least 12 SDRs, known as the retinol dehydrogenases (ROLDHs), have been identified (Liden et al., 2003). In cells, RAL is converted into RA by at least 4 enzymes in the aldehyde dehydrogenase (ADH) family known as the retinaldehyde dehydrogenases (RALDH) type 1-4 (Duester, 2001). Knock-out mice for RALDH2 display an embryonic lethal phenotype that is partially rescued by treatment with RA (Niederreither et al., 2001), suggesting that RA synthesized by the embryo is important for development *in-utero*.

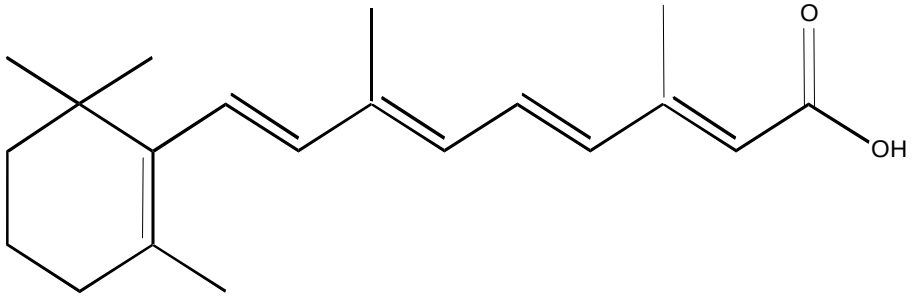


Figure 1.1 Retinoic acid

RA synthesis *in-utero* is required during embryonic development, but RA is also known to be toxic, as well as teratogenic, suggesting that maintenance of critical RA levels is important. RA is degraded in cells by cyp26, a member of the cytochrome P450 enzyme family (Ray et al., 1997; Sonneveld et al., 1998; Sonneveld and van der Saag, 1998). Cyp26 is also known as retinoic acid-induced P450 (P450RAI) and as its name implies, P450RAI expression has been shown to be greatly induced by RA in a number of cell types (Abu-Abed et al., 1998; de Roos et al., 1999; Marikar et al., 1998). At least three P450RAI retinoic acid degrading enzymes have been identified, known as cyp26a, b, and c. Knock-out mice lacking cyp26a1 expression die during mid-gestation and have a phenotype similar to that of mice treated with a teratogenic RA dose (Abu-Abed et al., 2001). Increasing evidence indicates that tight regulation of RA concentrations in cells is important, and support for this comes from the multiple and redundant RA synthesis and degradation pathways that have been identified.

Retinoic acid is a potent regulator of transcription, and it is well known that RA binds to and modulates the activities of ligand-inducible transcription factors known as the retinoic acid receptors (RARs) (Brand et al., 1990; Dalman et al., 1991; Nagpal et al., 1992). RA and its modulation of RAR activity have been shown to play a critical role in the differentiation of cells. RA has been shown to be required for limb regeneration in amphibians (Maden, 1997), and has been suggested to be able to promote the repair of damaged nerve tissue in mammalian whole-animal models (Zhelyaznik et al., 2003). Heart morphogenesis has also been shown to critically involve RA (Zhelyaznik et al., 2003). Chondrogenesis and skeletal progenitor cell differentiation have

been shown to be inhibited by RA ((Cho et al., 2003) and (Weston et al., 2002) respectively). RA and RAR have also been shown to inhibit the growth of a number of cancer cell models, including models for breast cancer (Darro et al., 1998) and cervical cancers (Behbakht et al., 1996). RA has been used with some success as a treatment for certain cancers (Shen et al., 2004; Soprano et al., 2004), but toxicity issues involved with RA treatment have limited its use.

1.2 NUCLEAR HORMONE RECEPTORS

It is known that small lipophylic compounds can act as signaling molecules in a variety of critical cellular processes. Many lipids, including long-chain fatty acids, prostaglandins, and retinoic acid, have been shown to play critical signaling roles in inflammation, cell growth, and differentiation. The wide range of processes that are effected by small lipophylic molecules come in part from their ability to act as signaling molecules that directly regulate gene expression. Many lipids and their metabolites have been shown to do so by acting as ligands that modulate the activity of transcription factors known as the nuclear hormone receptors.

The nuclear hormone receptors consist of both the steroid and the non-steroid hormone receptors. The steroid receptor subfamily includes the estrogen, androgen and glucocorticoid receptors. The non-steroid receptors, including the retinoic acid receptors (RAR) and peroxisome proliferator-activated receptors (PPAR) typically bind receptor-specific lipophylic ligands (Laudet and Gronemeyer, 2002).

The nuclear hormone receptors are a structurally related class of proteins, containing 5-6 structural domains (A-F) of specific function (fig. 1.2). The amino terminal A/B domain, which is highly variable between receptors, possesses a ligand independent transcriptional activity. The C domain consists of a DNA binding domain (DBD), which contains two highly conserved zinc finger DNA-binding motifs that bind to receptor-specific DNA response elements. While the steroid receptors typically bind to DNA as homodimers, the non-steroid receptors bind in a heterodimer with a common partner, the retinoid X receptor (RXR). The D domain is a small “hinge” region that connects the DBD to domain E, the ligand binding domain (LBD). The more variable LBD possesses a ligand-inducible transcriptional activity. The LBD is known to be important for receptor dimerization, as well as for protein-protein interactions with other transcriptional effectors. The C-terminal domain F is variable and does not exist in a number of the nuclear hormone receptors, and its function is not known.

The nuclear receptors bind to DNA as a dimer on a receptor-specific DNA response element. In the absence of ligands, nuclear receptors bind to corepressors. The corepressors down regulate the transcription of genes by recruiting proteins that modify chromatin, thereby inhibiting the binding of the general transcription machinery. When ligands bind to the receptors, a structural change occurs in the protein, and they release the corepressors. At the same time, the receptors gain an affinity for coactivator proteins, which induce chromatin to become accessible to the general transcription machinery, thereby upregulating gene expression (Bastien and Rochette-Egly, 2004; Dilworth and Chambon, 2001; Kinyamu and Archer, 2004; Xu et al., 1999).

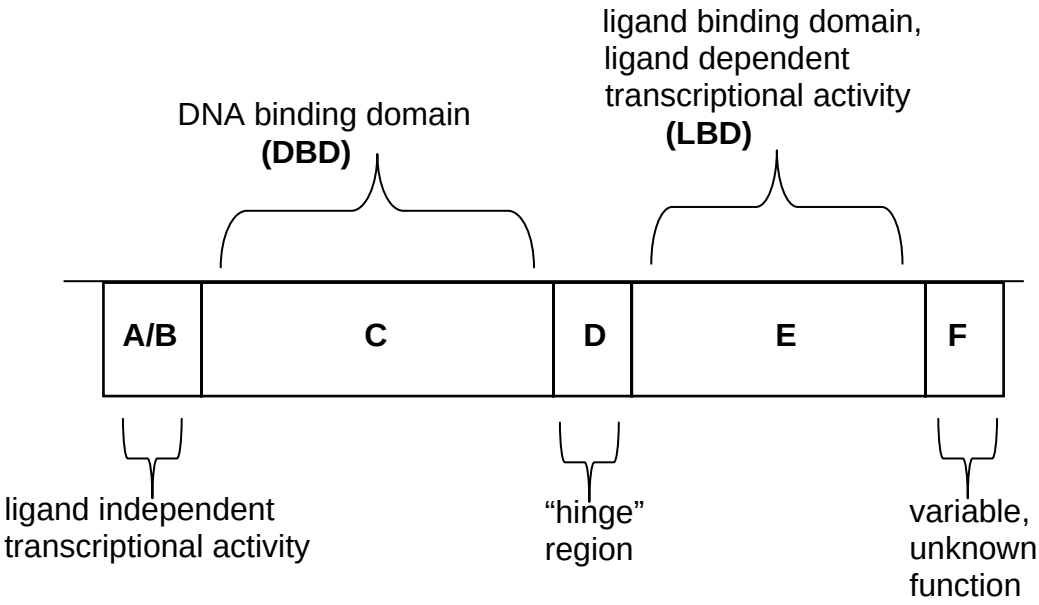


Figure 1.2 Domain structure of the nuclear hormone receptors and associated functions.

Several nuclear hormone receptors are regulated by fatty acids and retinoids, including the peroxisome proliferator activated receptors (PPARs) and the retinoic acid receptors (RARs), among others. PPARs, which are activated by fatty acids and some of their metabolites, (Desvergne and Wahli, 1999; Forman et al., 1997; Kliewer et al., 1997) are known to play roles in a number of critical cellular processes, and have been shown to play a role in the onset of diabetes and obesity (Kersten, 2002). Drugs designed as PPAR-specific ligands are currently used to treat diabetes (Carey et al., 2002; Spiegelman, 1998; Tiikkainen et al., 2004). Retinoic acid receptors (RARs) are activated by retinoic acid (Brand et al., 1990; Dalman et al., 1991; Nagpal et al., 1992). Knock-out mice for RARs suggest that their function is important for development (Lohnes et al., 1995; Mendelsohn et al., 1994).

1.3 THE CELLULAR RETINOIC ACID BINDING PROTEINS

Retinoic acid binds to and modulates the activities of RARs, but it is also known to bind to the cellular retinoic acid binding proteins (CRABP-I and CRABP-II) with a high affinity (Dong et al., 1999). CRABPs are members of a family of small structurally similar cytosolic proteins known as the intracellular lipid binding proteins (iLBPs). The iLBPs are not very well conserved in their primary sequences, but share a very similar overall three-dimensional structure. The iLBPs have a characteristic lipid binding cavity that is comprised of a lipid-binding pocket created by two orthogonal 5-stranded beta sheets, which is covered by a characteristic helix-loop-helix “lid” (figure 1.3)

(Gutierrez-Gonzalez et al., 2002; Kleywegt et al., 1994; Veerkamp and Maatman, 1995).

The iLBPs are thought to function as shuttling proteins that bind to fatty acids and their metabolites and protect them in the cytosol. Studies suggest that some FABPs pull their ligands from membranes by a collision-mediated process (Thumser and Storch, 2000). A number of studies suggest that certain FABPs deliver their ligands to membranes by a similar mechanism (Hsu and Storch, 1996; Kim and Storch, 1992; Wootan et al., 1993) Intestinal fatty acid binding protein (I-FABP) is an iLBP that is highly expressed in enterocytes. I-FABP has been demonstrated to bind certain non-fatty acid lipophilic drugs, and I-FABP has been suggested to increase their cytosolic solubility, facilitating their transport from the intestinal lumen across the enterocyte (Velkov et al., 2005).

While many iLBPs, including I-FABP, bind to a variety of fatty acids and their metabolites (Hanhoff et al., 2002; Norris and Spector, 2002; Widstrom et al., 2001), the CRABPs bind to RA with a high selectivity, specificity and affinity (Dong et al., 1999).

There are two known CRABPs in mammalian cells, CRABP-I and CRABP-II. They are distinct gene products but share a very high sequence homology (Figure 1.4). CRABP orthologs have been found in mammals, birds, fishes, and amniotes. Both CRABP transcripts are expressed in the embryo of mice in a specific, non-overlapping and stage dependant pattern (Ruberte et al., 1992), suggesting that they play distinct biological roles.

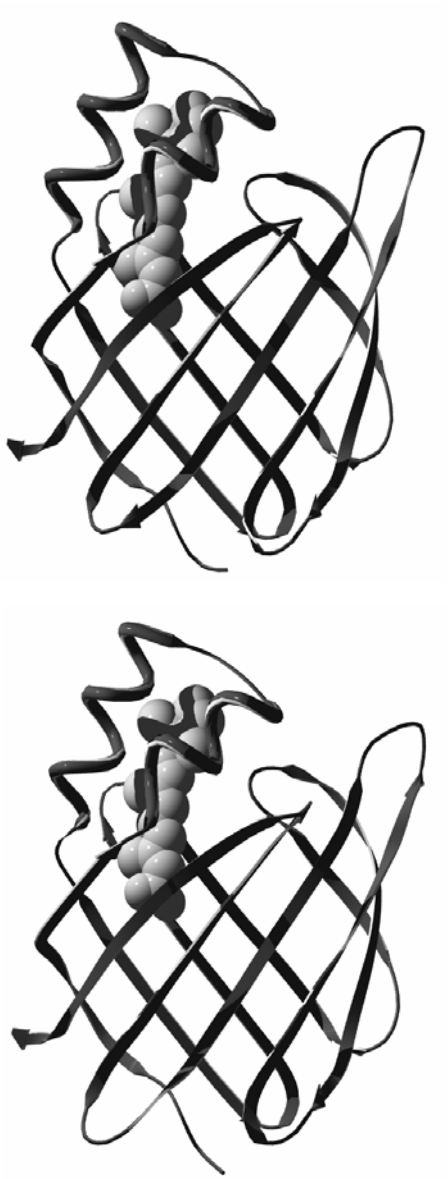


Figure 1.3 Crystal structures of holo- cellular retinoic acid binding proteins type I (top) and type II (Kleywegt et al., 1994).

A number of reports from *in vitro* and *in vivo* studies have suggested that the induction of CRABP-II expression is concurrent with enzymes involved with the synthesis of RA (Bucco et al., 1997; Li et al., 2004). CRABP-I has been suggested to play a role in promoting the degradation of RA in some cells (Boylan and Gudas, 1991; Won et al., 2004). The unique expression patterns and evolutionary conservation of the CRABPs suggest that they play important roles in the biological activities of RA. KO mice for either CRABP-II, or for both CRABP-I and II, have been created (Lampron et al., 1995).

Interestingly, the only phenotype was a relatively mild digit malformation that was manifest in a small percentage of offspring.CRABP-II KOs showed an occasional phenotype, and the CRABP-I/II double KO mice had an increased percentage of offspring with the same digit malformation. None of the mice were found to be infertile, and they developed normally. These data suggest a non-critical role for the CRABPs. The emergence of a phenotype may result if mothers were compromised in their vitamin A status, but such dietary effects have yet to be investigated.

Retinoic acid is known to be a potent activator of transcription as an activator of RAR. It has been suggested that there is difference between CRABP-I and CRABP-II in their abilities to modulate RA-induced RAR activities (Dong et al., 1999). In a cell model system, ectopic CRABP-I expression has no significant effects on RAR activity in response to RA treatment, where as ectopic CRABP-II expression resulted in a significant increase in the ability of RA to stimulate RAR activity. It has also been found that CRABP-II is mainly cytosolic in the absence of RA, and that upon RA treatment CRABP-II accumulates in the nucleus (Budhu and Noy, 2002).

```
          1                                                  50
 CRABP-I  MPNFAGTWKM RSSENFDELL KALGVNAMLR KVAVAAASKP HVEIRQDGDQ
CRABP-II  MPNFSGNWKI IRSENFEELL KVLGVNVMLR KIAVAAASKP AVEIKQEGDT

          51                                                 100
 CRABP-I  FYIKTSTTVR TTEINFKVGE GFEEETVDGR KCRSLATWEN ENKIHCTQTL
CRABP-II  FYIKTSTTVR TTEINFKVGE EFEEQTVDGR PCKSLVKWES ENKMVCEQKL

          101                                 138
 CRABP-I  LEGDGPKTYW TRELAND.EL ILTFGADDVV CTRIYVRE
CRABP-II  LKGEGPKTSW TRELTNDGEL ILTMTADDVV CTRVYVRE
```

Figure 1.4 Alignment of human CRABP-I and CRABP-II amino acid sequences. Sequence homology is in bold print. Alignment was done using Multalin (Corpet, 1988) and accession #'s NM_004378 (CRABP-I) and # NM_001878 (CRABP-II).

The CRABPs, like other iLBPs, are believed to transport their labile ligands through the cytoplasm of cell. CRABP-II is thought to enhance RAR transcriptional activities by enhancing the movement of RA into the nucleus, where RAR functions as a transcription factor (Bastie et al., 2001; Budhu et al., 2001; Budhu and Noy, 2002).

1.4 OVERALL RESEARCH GOAL

Previous work has shown that CRABP-I and CRABP-II differ in their ability to modulate the transcriptional activities of RAR. Expression of CRABP-II appears to dramatically enhance RA-induced RAR transcriptional activity in cells while CRABP-I does not. CRABP-II has also been suggested to "channel" retinoic acid to RAR through a transient protein-protein interaction *in*-vitro, while CRABP-I does not. CRABP-I and CRABP-II are both cytosolic in the absence of RA, and while CRABP-II is localized to the nucleus in response to RA binding, CRABP-I remains in the cytoplasm.

The work presented in this thesis was undertaken in order to further investigate the biological role of CRABP-II. The goal of these studies was to investigate the mechanisms for RA-induced changes in the subcellular localization of CRABP-II.

1.5 REFERENCES

Abu-Abed, S., Dolle, P., Metzger, D., Beckett, B., Chambon, P., and Petkovich, M. (2001). The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. Genes Dev *15*, 226-240.

Abu-Abed, S. S., Beckett, B. R., Chiba, H., Chithalen, J. V., Jones, G., Metzger, D., Chambon, P., and Petkovich, M. (1998). Mouse P450RAI (CYP26) expression and retinoic acid-inducible retinoic acid metabolism in F9 cells are regulated by retinoic acid receptor gamma and retinoid X receptor alpha. J Biol Chem *273*, 2409-2415.

Bastie, J. N., Despouy, G., Balitrand, N., Rochette-Egly, C., Chomienne, C., and Delva, L. (2001). The novel co-activator CRABPII binds to RARalpha and RXRalpha via two nuclear receptor interacting domains and does not require the AF-2 'core'. FEBS Lett *507*, 67-73.

Bastien, J., and Rochette-Egly, C. (2004). Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene *328*, 1-16.

Behbakht, K., DeGeest, K., Turyk, M. E., and Wilbanks, G. D. (1996). All-trans-retinoic acid inhibits the proliferation of cell lines derived from human cervical neoplasia. Gynecol Oncol *61*, 31-39.

Boylan, J. F., and Gudas, L. J. (1991). Overexpression of the cellular retinoic acid binding protein-I (CRABP-I) results in a reduction in differentiation-specific gene expression in F9 teratocarcinoma cells. J Cell Biol *112*, 965-979.

Brand, N. J., Petkovich, M., and Chambon, P. (1990). Characterization of a functional promoter for the human retinoic acid receptor-alpha (hRAR-alpha). Nucleic Acids Res *18*, 6799-6806.

Bucco, R. A., Zheng, W. L., Davis, J. T., Sierra-Rivera, E., Osteen, K. G., Chaudhary, A. K., and Ong, D. E. (1997). Cellular retinoic acid-binding protein(II) presence in rat uterine epithelial cells correlates with their synthesis of retinoic acid. Biochemistry *36*, 4009-4014.

Budhu, A., Gillilan, R., and Noy, N. (2001). Localization of the RAR interaction domain of cellular retinoic acid binding protein-II. J Mol Biol *305*, 939-949.

Budhu, A. S., and Noy, N. (2002). Direct channeling of retinoic acid between cellular retinoic acid-binding protein II and retinoic acid receptor sensitizes mammary carcinoma cells to retinoic acid-induced growth arrest. Mol Cell Biol *22*, 2632-2641.

Carey, D. G., Cowin, G. J., Galloway, G. J., Jones, N. P., Richards, J. C., Biswas, N., and Doddrell, D. M. (2002). Effect of rosiglitazone on insulin sensitivity and body composition in type 2 diabetic patients [corrected]. Obes Res *10*, 1008-1015.

Cavazzini, D., Catizone, A., Galdieri, M., and Ottonello, S. (2003). Vitamin A metabolism in cultured somatic cells from rat testis. Mol Cell Biochem *252*, 165-171.

Cho, S. H., Oh, C. D., Kim, S. J., Kim, I. C., and Chun, J. S. (2003). Retinoic acid inhibits chondrogenesis of mesenchymal cells by sustaining expression of N-cadherin and its associated proteins. J Cell Biochem *89*, 837-847.

Dalman, F. C., Sturzenbecker, L. J., Levin, A. A., Lucas, D. A., Perdew, G. H., Petkovitch, M., Chambon, P., Grippo, J. F., and Pratt, W. B. (1991). Retinoic acid receptor belongs to a subclass of nuclear receptors that do not form "docking" complexes with hsp90. Biochemistry *30*, 5605-5608.

Darro, F., Cahen, P., Vianna, A., Decaestecker, C., Nogaret, J. M., Leblond, B., Chaboteaux, C., Ramos, C., Petein, M., Budel, V.*, et al.* (1998). Growth inhibition of human in vitro and mouse in vitro and in vivo mammary tumor models by retinoids in comparison with tamoxifen and the RU-486 anti-progestagen. Breast Cancer Res Treat *51*, 39-55.

de Roos, K., Sonneveld, E., Compaan, B., ten Berge, D., Durston, A. J., and van der Saag, P. T. (1999). Expression of retinoic acid 4-hydroxylase (CYP26) during mouse and Xenopus laevis embryogenesis. Mech Dev *82*, 205-211.

Desvergne, B., and Wahli, W. (1999). Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev *20*, 649-688.

Dilworth, F. J., and Chambon, P. (2001). Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. Oncogene *20*, 3047-3054.

Dong, D., Ruuska, S. E., Levinthal, D. J., and Noy, N. (1999). Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. J Biol Chem *274*, 23695-23698.

Duester, G. (2001). Genetic dissection of retinoid dehydrogenases. Chem Biol Interact *130-132*, 469-480.

Forman, B. M., Chen, J., and Evans, R. M. (1997). Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A *94*, 4312-4317.

Gutierrez-Gonzalez, L. H., Ludwig, C., Hohoff, C., Rademacher, M., Hanhoff, T., Ruterjans, H., Spener, F., and Lucke, C. (2002). Solution structure and backbone dynamics of human epidermal-type fatty acid-binding protein (E-FABP). Biochem J *364*, 725-737.

Hanhoff, T., Lucke, C., and Spener, F. (2002). Insights into binding of fatty acids by fatty acid binding proteins. Mol Cell Biochem *239*, 45-54.

Hsu, K. T., and Storch, J. (1996). Fatty acid transfer from liver and intestinal fatty acid-binding proteins to membranes occurs by different mechanisms. J Biol Chem *271*, 13317-13323.

Kersten, S. (2002). Peroxisome proliferator activated receptors and obesity. Eur J Pharmacol *440*, 223-234.

Kim, H. K., and Storch, J. (1992). Mechanism of free fatty acid transfer from rat heart fatty acid-binding protein to phospholipid membranes. Evidence for a collisional process. J Biol Chem *267*, 20051-20056.

Kinyamu, H. K., and Archer, T. K. (2004). Modifying chromatin to permit steroid hormone receptor-dependent transcription. Biochim Biophys Acta *1677*, 30-45.

Kleywegt, G. J., Bergfors, T., Senn, H., Le Motte, P., Gsell, B., Shudo, K., and Jones, T. A. (1994). Crystal structures of cellular retinoic acid binding proteins I and II in complex with all-trans-retinoic acid and a synthetic retinoid. Structure *2*, 1241-1258.

Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997). Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci U S A *94*, 4318-4323.

Lampron, C., Rochette-Egly, C., Gorry, P., Dolle, P., Mark, M., Lufkin, T., LeMeur, M., and Chambon, P. (1995). Mice deficient in cellular retinoic acid binding protein II (CRABPII) or in both CRABPI and CRABPII are essentially normal. Development *121*, 539-548.

Laudet, V., and Gronemeyer, H. (2002). The NuclearReceptor FactsBook (London and San Diego, Academic Press).

Li, X. H., Kakkad, B., and Ong, D. E. (2004). Estrogen directly induces expression of retinoic acid biosynthetic enzymes, compartmentalized between the epithelium and underlying stromal cells in rat uterus. Endocrinology *145*, 4756-4762.

Liden, M., Tryggvason, K., and Eriksson, U. (2003). Structure and function of retinol dehydrogenases of the short chain dehydrogenase/reductase family. Mol Aspects Med *24*, 403-409.

Lohnes, D., Mark, M., Mendelsohn, C., Dolle, P., Decimo, D., LeMeur, M., Dierich, A., Gorry, P., and Chambon, P. (1995). Developmental roles of the retinoic acid receptors. J Steroid Biochem Mol Biol *53*, 475-486.

Maden, M. (1997). Retinoic acid and its receptors in limb regeneration. Semin Cell Dev Biol *8*, 445-453.

Marikar, Y., Wang, Z., Duell, E. A., Petkovich, M., Voorhees, J. J., and Fisher, G. J. (1998). Retinoic acid receptors regulate expression of retinoic acid 4-hydroxylase that specifically inactivates all-trans retinoic acid in human keratinocyte HaCaT cells. J Invest Dermatol *111*, 434-439.

Mendelsohn, C., Lohnes, D., Decimo, D., Lufkin, T., LeMeur, M., Chambon, P., and Mark, M. (1994). Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. Development *120*, 2749-2771.

Nagpal, S., Saunders, M., Kastner, P., Durand, B., Nakshatri, H., and Chambon, P. (1992). Promoter context- and response element-dependent specificity of the transcriptional activation and modulating functions of retinoic acid receptors. Cell *70*, 1007-1019.

Napoli, J. L. (1999). Retinoic acid: its biosynthesis and metabolism. Prog Nucleic Acid Res Mol Biol *63*, 139-188.

Niederreither, K., Vermot, J., Messaddeq, N., Schuhbaur, B., Chambon, P., and Dolle, P. (2001). Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. Development *128*, 1019-1031.

Norris, A. W., and Spector, A. A. (2002). Very long chain n-3 and n-6 polyunsaturated fatty acids bind strongly to liver fatty acid-binding protein. J Lipid Res *43*, 646-653.

Ray, W. J., Bain, G., Yao, M., and Gottlieb, D. I. (1997). CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. J Biol Chem *272*, 18702-18708.

Ruberte, E., Friederich, V., Morriss-Kay, G., and Chambon, P. (1992). Differential distribution patterns of CRABP I and CRABP II transcripts during mouse embryogenesis. Development *115*, 973-987.

Shen, Z. X., Shi, Z. Z., Fang, J., Gu, B. W., Li, J. M., Zhu, Y. M., Shi, J. Y., Zheng, P. Z., Yan, H., Liu, Y. F.*, et al.* (2004). All-trans retinoic acid/As2O3 combination yields a high quality remission and survival in newly diagnosed acute promyelocytic leukemia. Proc Natl Acad Sci U S A *101*, 5328-5335.

Sonneveld, E., van den Brink, C. E., van der Leede, B. M., Schulkes, R. K., Petkovich, M., van der Burg, B., and van der Saag, P. T. (1998). Human retinoic acid (RA) 4-hydroxylase (CYP26) is highly specific for all-trans-RA and can be induced through RA receptors in human breast and colon carcinoma cells. Cell Growth Differ *9*, 629-637.

Sonneveld, E., and van der Saag, P. T. (1998). Metabolism of retinoic acid: implications for development and cancer. Int J Vitam Nutr Res *68*, 404-410.

Soprano, D. R., Qin, P., and Soprano, K. J. (2004). Retinoic Acid Receptors and Cancers. Annu Rev Nutr *24*, 201-221.

Spiegelman, B. M. (1998). PPAR-gamma: adipogenic regulator and thiazolidinedione receptor. Diabetes *47*, 507-514.

Thumser, A. E., and Storch, J. (2000). Liver and intestinal fatty acid-binding proteins obtain fatty acids from phospholipid membranes by different mechanisms. J Lipid Res *41*, 647-656.

Tiikkainen, M., Hakkinen, A. M., Korsheninnikova, E., Nyman, T., Makimattila, S., and Yki-Jarvinen, H. (2004). Effects of rosiglitazone and metformin on liver fat content, hepatic insulin resistance, insulin clearance, and gene expression in adipose tissue in patients with type 2 diabetes. Diabetes *53*, 2169-2176.

Veerkamp, J. H., and Maatman, R. G. (1995). Cytoplasmic fatty acid-binding proteins: their structure and genes. Prog Lipid Res *34*, 17-52.

Velkov, T., Chuang, S., Wielens, J., Sakellaris, H., Charman, W. N., Porter, C. J., and Scanlon, M. J. (2005). The interaction of lipophilic drugs with intestinal fatty acid binding protein. J Biol Chem.

Weston, A. D., Chandraratna, R. A., Torchia, J., and Underhill, T. M. (2002). Requirement for RAR-mediated gene repression in skeletal progenitor differentiation. J Cell Biol *158*, 39-51.

Widstrom, R. L., Norris, A. W., and Spector, A. A. (2001). Binding of cytochrome P450 monooxygenase and lipoxygenase pathway products by heart fatty acid-binding protein. Biochemistry *40*, 1070-1076.

Won, J. Y., Nam, E. C., Yoo, S. J., Kwon, H. J., Um, S. J., Han, H. S., Kim, S. H., Byun, Y., and Kim, S. Y. (2004). The effect of cellular retinoic acid binding protein-I expression on the CYP26-mediated catabolism of all-trans retinoic acid and cell proliferation in head and neck squamous cell carcinoma. Metabolism *53*, 1007-1012.

Wootan, M. G., Bernlohr, D. A., and Storch, J. (1993). Mechanism of fluorescent fatty acid transfer from adipocyte fatty acid binding protein to membranes. Biochemistry *32*, 8622-8627.

Xu, L., Glass, C. K., and Rosenfeld, M. G. (1999). Coactivator and corepressor complexes in nuclear receptor function. Curr Opin Genet Dev *9*, 140-147.

Zhelyaznik, N., Schrage, K., McCaffery, P., and Mey, J. (2003). Activation of retinoic acid signalling after sciatic nerve injury: up-regulation of cellular retinoid binding proteins. Eur J Neurosci *18*, 1033-1040.

CHAPTER TWO

*A LIGAND-ACTIVATED NUCLEAR LOCALIZATION SIGNAL IN CELLULAR RETINOIC ACID BINDING PROTEIN-II

*Reprinted from Molecular Cell, Volume 18(3), Sessler, R.J. and Noy, N., A ligand-activated nuclear localization signal in cellular retinoic acid binding protein-II, Pages No. 343-53, Copyright 2005, with permission from Elsevier.

2.1 SUMMARY

Primary sequences of proteins often contain motifs that serve as ‘signatures’ for subcellular targeting, such as a nuclear localization signal (NLS). However, many nuclear proteins do not harbor a recognizable NLS and the pathways that mediate their nuclear translocation are unknown. This work focuses on CRABP-II, a cytosolic protein that moves to the nucleus upon binding of retinoic acid. While CRABP-II does not contain an NLS in its primary sequence, such a motif could be recognized in the protein’s tertiary structure. We map the retinoic acid-induced structural rearrangements that result in the presence of this NLS in holo- but not apo-CRABP-II. The signal, whose three-dimensional configuration aligns strikingly well with a ‘classical’ NLS, mediates ligand-induced association of CRABP-II with importinα and is critical for nuclear localization of the protein. The ligand-controlled NLS “switch” of CRABP-II may represent a general mechanism for post-translational regulation of the subcellular distribution of a protein.

2.2 INTRODUCTION

Small lipophilic compounds often play important roles in regulating biological functions. Many such molecules, e.g. retinoic acid, long chain fatty acids and eicosanoids, function by activating ligand-inducible transcription factors that are members of the super-family of nuclear hormone receptors (Laudet and Gronemeyer, 2002). Hence, retinoic acid activates retinoic acid receptors (RAR) (Dalman et al., 1991; Nagpal et al., 1992) while fatty acids and some of their metabolites activate peroxisome proliferator-activated

receptors (PPAR) (Desvergne and Wahli, 1999; Forman et al., 1997; Kliewer et al., 1997). Lipophilic ligands thus regulate the expression of genes that control various aspects of cellular metabolism, proliferation, and differentiation. Type II nuclear receptors, including RAR and PPAR, bind to DNA response elements as heterodimers with a common partner, the retinoid X receptor, and regulate transcription in response to their cognate ligands (Aranda and Pascual, 2001; Chambon, 1996; Laudet and Gronemeyer, 2002). While the mechanisms by which ligands modulate the activities of nuclear receptors have become increasingly clear (Bastien and Rochette-Egly, 2004; Dilworth and Chambon, 2001; Xu et al., 1999), an important question that remains open is how these ligands, which are poorly soluble and often labile in aqueous environments, traverse the cytoplasm, enter the nucleus, and reach their cognate receptors.

In addition to nuclear receptors, many lipophilic compounds bind in cells to intracellular lipid binding proteins (iLBPs), a family of small (~15 kDa) proteins that share a remarkably similar β-clam structure comprised of two 5-stranded orthogonal β-sheets that form a ligand binding pocket, and a single helix-loop-helix “lid” that appears to limit ligand access (Gutierrez-Gonzalez et al., 2002; Kleywegt et al., 1994; Veerkamp and Maatman, 1995). Although similar in structure, iLBPs diverge in their primary sequences and they bind lipophilic molecules with distinct selectivities. Cellular retinol binding proteins (CRBP-I and II) bind retinol and retinaldehyde with high specificity (Levin et al., 1988; Noy and Blaner, 1991), and cellular retinoic acid binding proteins (CRABP-I and II) associate with retinoic acid with subnanomolar affinities (Dong et al., 1999). In contrast, other iLBPs, known as fatty acid binding proteins (FABPs), display broad selectivities and bind a variety of ligands with

similar affinities (Hanhoff et al., 2002; Norris and Spector, 2002; Widstrom et al., 2001).

The evolutionary conservation of iLBPs suggests that they play important roles in the biological activities of their ligands but these functions remain incompletely understood. These proteins are thought to act as carriers for their ligands, serving to solubilize and protect them in the aqueous environment of the cytosol. In addition to this general role, it has been suggested that particular iLBPs may have specific functions. It was proposed that some iLBPs facilitate the movement of fatty acids into membranes (Kim and Storch, 1992; Wootan et al., 1993), that K-FABP interacts with the calcium binding protein psoriasin (Hagens et al., 1999a; Hagens et al., 1999b), and that A-FABP and K-FABP directly associate with hormone sensitive lipase (Jenkins-Kruchten et al., 2003; Shen et al., 2001). The structural basis for and the functional consequence of these interactions remain to be clarified.

Our recent studies demonstrated that three iLBPs play selective roles in enhancing the transcriptional activities of nuclear receptors with which they share a common ligand. Specifically, we showed that CRABP-II, K-FABP and A-FABP enhance the ligand-induced transcriptional activities of RARα, PPARβ/δ and PPARγ, respectively. The data established that these iLBPs exert their effects by directly interacting with the respective receptors, and that the resulting complexes mediate “channeling” of ligands to the receptors, thereby facilitating their ligation and enhancing their transcriptional activities (Budhu and Noy, 2002; Dong et al., 1999; Tan et al., 2002).

Direct interactions between iLBPs and nuclear receptors require that the two types of proteins colocalize in the same subcellular compartment at least under some conditions. This is a non-trivial issue because nuclear

receptors function in the nucleus while iLBPs are usually believed to be cytosolic. Studies of the subcellular localization of CRABP-II, K-FABP and A-FABP revealed that these proteins are indeed cytosolic in the absence of ligands, but that they translocate to the nucleus upon treatment of cells with appropriate ligands (Budhu and Noy, 2002; Tan et al., 2002). These observations raise the question of the structural basis for the ligand-induced nuclear localization of these iLBPs.

The best characterized nuclear localization signal (NLS) of proteins is exemplified by the 'classical' NLS of the SV40-T antigen which is identifiable in the primary sequence of a protein as a series of basic residues in the form K(K/R)X(K/R) (Chook and Blobel, 2001; Dingwall and Laskey, 1991; Hodel et al., 2001; Kalderon et al., 1984; Robbins et al., 1991). Such an NLS is often recognized by adapter proteins known as α-importins which, in turn, associate with one of the various forms of importinβ to mediate nuclear localization (Chook and Blobel, 2001). It has also been reported that importinβ can interact directly with some cargoes. While nuclear targeting sequences recognized by importinβ are less well understood and may vary, it has been shown that, in some cases, such sequences are reminiscent of 'classical' NLS in that they are comprised of regions rich in basic residues (Palmeri and Malim, 1999; Truant and Cullen, 1999).

Here, we undertook to identify the region that mediates the ligand-induced nuclear localization of CRABP-II, a protein which, like other iLBPs, does not contain any recognizable NLS in its primary sequence. Considering that CRABP-II moves to the nucleus only when ligated, we reasoned that ligand-binding must induce a conformational change that enables nuclear targeting. Comparison of the electrostatic surface potentials of apo- and holo-

CRABP-II revealed a positively-charged surface patch which is present in the holo- but not the apo-protein. We show that this patch is comprised of three basic residues whose configuration in the tertiary structure of holo-CRABP-II is similar to the 3-dimensional configuration of a 'classical' NLS. We show further that this motif is essential for association of CRABP-II with importin hSRP1 as well as for the protein's ligand-induced nuclear localization. The data thus demonstrate the presence of a ligand-controlled NLS "switch" that is manifested in the tertiary, but not the primary, structure of CRABP-II.

2.3 RESULTS

2.3.1 CRABP-II accumulates in the nucleus in response to RA.

An important step in the process that enables CRABP-II to enhance the transcriptional activity of RAR (Budhu et al., 2001; Budhu and Noy, 2002; Dong et al., 1999) is a ligand-induced nuclear translocation of the binding protein. Such a response was previously shown to take place in COS-7 cells (Budhu and Noy, 2002). To examine whether RA-induced nuclear targeting is a general feature of CRABP-II, we used MCF-7 mammary carcinoma cells, which endogenously express this protein at a level of over an order of magnitude higher than COS-7 cells (data not shown). MCF-7 cells were maintained in delipidated serum to deplete vitamin A and RA pools, treated with RA for 30 min., fixed, immunostained with CRABP-II antibodies and imaged by confocal fluorescence microscopy (Fig. 2.1).

Similarly to its localization in COS-7 cells, CRABP-II in untreated MCF-7 cells was found to be extra-nuclear (Fig. 2.1A-2.1C). In contrast with the apparent uniform distribution of unligated CRABP-II in cytosol of COS-7 cells (Budhu and Noy, 2002), CRABP-II in untreated MCF-7 cells accumulated in the perinuclear region where it displayed a punctate pattern, suggesting that it is associated with specific subcellular organelle(s). The differences in the appearance of apo-CRABP-II in the two cell types could relate to variations in its expression levels or to differential expression of binding partners. Nevertheless, similarly to its response in COS-7 cells, CRABP-II in RA-treated MCF-7 cells was found almost exclusively in the nucleus (Fig. 2.1D-2.1F.). Hence, ligand-induced nuclear localization is a general feature of CRABP-II.

To further examine the subcellular localization of CRABP-II and to enable subsequent mutagenesis analyses using live cells, a construct of the protein fused to green fluorescent protein (GFP) was used. COS-7 cells were transfected with an expression vector encoding GFP-tagged CRABP-II and cells were imaged prior to or following a 30 min. treatment with RA. As a control, cells were transfected with a plasmid expressing GFP alone. GFP was distributed across the cells (Fig. 2.2A), most likely reflecting that its high expression level resulted in leakage into the nucleus even in the absence of a specific NLS. The distribution of GFP was not affected by RA treatment (Fig. 2.2B). Similarly, the high level of expression of GFP-tagged CRABP-II in untreated cells resulted in a distribution in the cytosol as well as the nucleus and in loss of the punctate perinuclear pattern observed for endogenous CRABP-II (compare Fig. 2.2C with Fig. 2.1A).

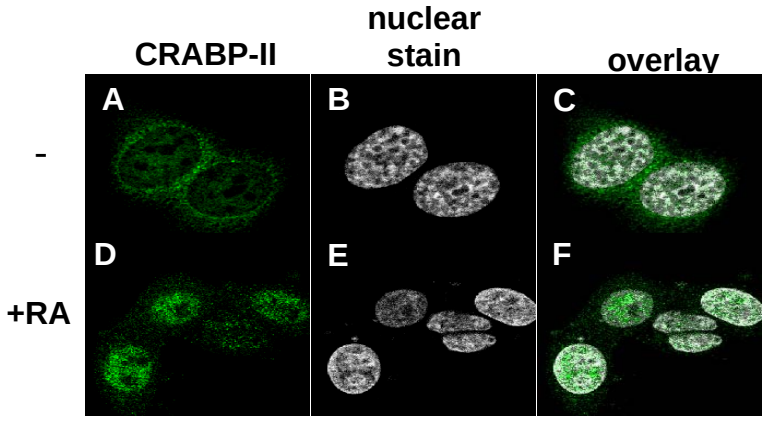


Figure 2.1. CRABP-II localizes to the nucleus in response to RA. MCF-7 cells were treated with ethanol (A-C) or 100 nM RA (D-F) for 30 min. (A,D) Immunostaining with CRABP-II antibodies; (B,E) Imaging of nuclei using the nucleic acid stain ToPro3; (C,F) Overlay of images of CRABP-II and nuclei.

Nevertheless, like the behavior of endogenously-expressed CRABP-II, addition of RA led to accumulation of GFP-CRABP-II in the nucleus, a process that appeared to be complete within 30 min. (Fig. 2.2D). Hence, while over-expression GFP-CRABP-II somewhat masked the proper distribution of the unligated protein, the construct properly underwent a ligand-induced nuclear translocation.

2.3.2 RA induces nuclear import of CRABP-II.

Accumulation of CRABP-II in the nucleus in response to RA may reflect ligand-induced nuclear import. Alternatively, RA may inhibit the nuclear export of the protein. To distinguish between these possibilities, the localization of CRABP-II was examined in the presence of leptomycin B (LMB), a compound that inhibits nuclear export mediated by nuclear export signals (NES). To verify the efficacy of LMB treatment, we generated a CRABP-II construct comprised of CRABP-II fused to an NES of the sequence MDLCQAFSDVILAEF, which mediates active export from the nucleus by the CRM-1 export machinery (Moroianu, 1999; Sweitzer et al., 2000). NES-GFP-CRABP-II was excluded from the nucleus both in the absence and in the presence of RA (Fig. 2.2E and 2.2F). In contrast, in cells treated with LMB, CRABP-II harboring the NES was distributed across the cells in the absence of RA, and translocated into nucleus in the presence of RA (Fig. 2.2G and 2.2H). Similarly, RA treatment resulted in nuclear accumulation of WT GFP-CRABP-II regardless of the presence of LMB (Fig. 2.2I and 2.2J). Hence, RA triggers nuclear accumulation of CRABP-II by inducing its nuclear import and not by inhibiting nuclear export.

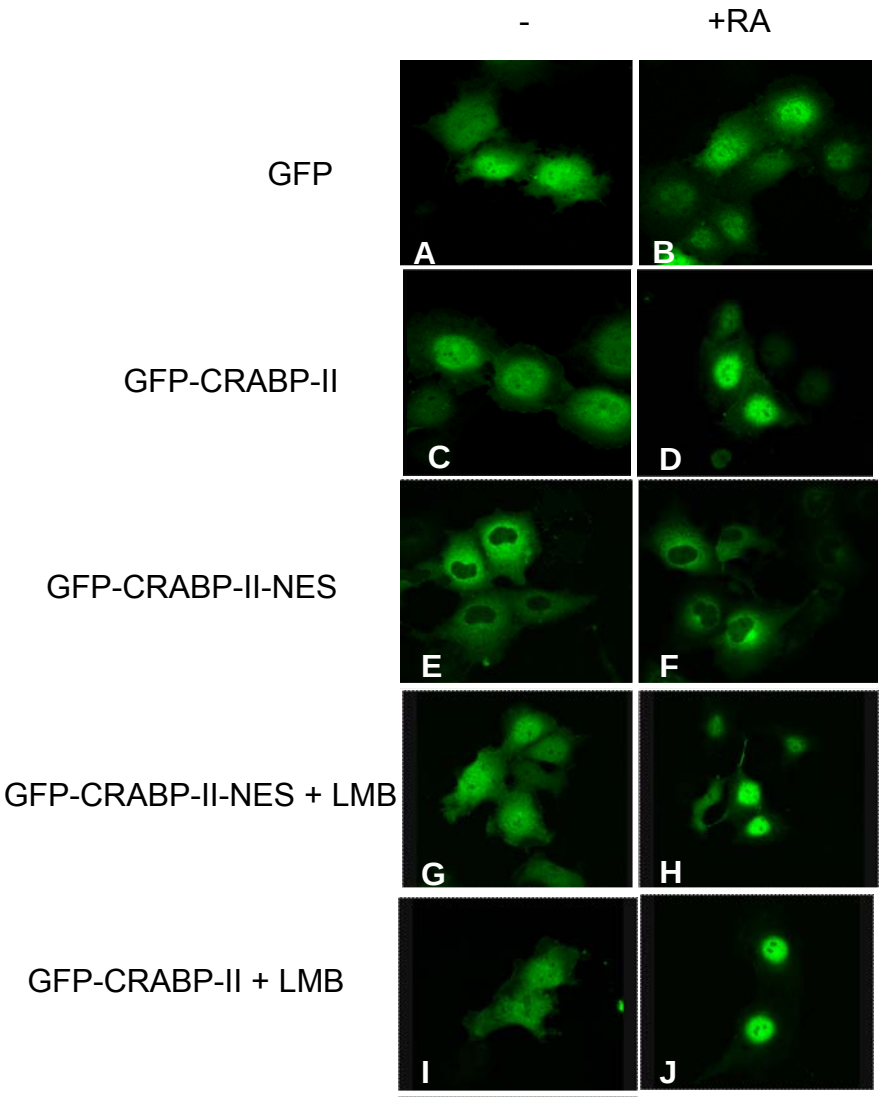
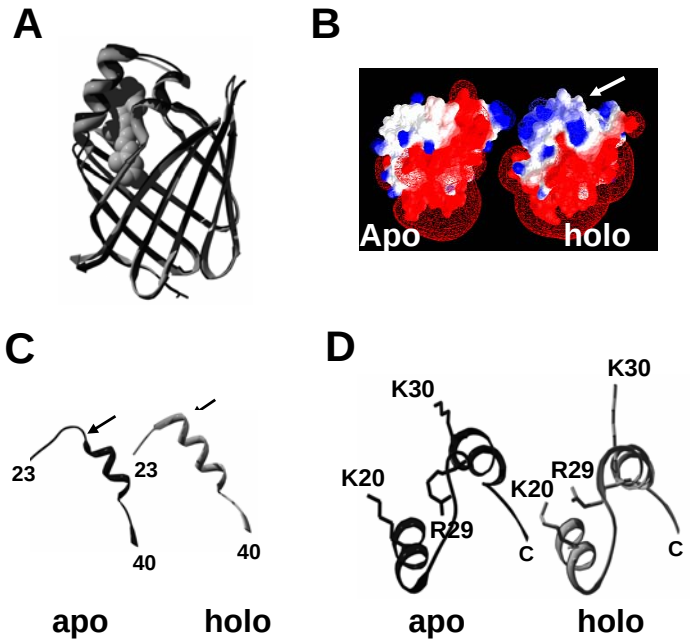


Figure 2.2. RA induces nuclear import of GFP-CRABP-II. COS-7 cells were transfected with expression vectors harboring the denoted CRABP-II constructs. Images of GFP-tagged proteins were acquired from live cells before (A,C,E,G,I) or after (B,D,F,H,J) a 30 min. treatment with RA (1 μM). Treatment with leptomycin B (LMB, 5 ng/ml) was carried out for 5 hours prior to and throughout imaging.

2.3.3 Identification of a putative NLS in holo-CRABP-II.

We then set out to delineate the structural basis for RA-induced nuclear targeting of CRABP-II. As the primary sequence of CRABP-II does not harbor any recognizable NLS sequence, we considered that the observations that nuclear targeting of the protein is controlled by RA imply that ligand-binding must induce a conformational change that results in recognition by nuclear import protein(s). Examination of the X-ray crystal structures of apo- and holo-CRABP-II (Chen et al., 1998; Kleywegt et al., 1994) showed however that the two structures are virtually super-imposable when viewed as ribbon models (Fig. 2.3A). We then compared the combined electrostatic surface potentials and molecular surfaces of the two forms of the protein. The comparison (Fig. 2.3B) revealed a surface patch in the helix-loop-helix region of CRABP-II whose electrostatic potential is neutral in apo-CRABP-II and becomes basic in the holo-protein. This shift could be mapped to the protein's second helix, a region that was previously noted to undergo a small structural rearrangement upon ligand binding (Chen et al., 1998; Wang and Yan, 1999). Specifically, the N-terminal of helix 2, which is loose in apo-CRABP-II, tightens to become part of the helix in the holo-protein (Fig. 2.3C). Consequently, two basic residues, R29 and K30, which are directed inward in apo-CRABP-II, rotate towards the surface in the holo-form (Fig. 2.3D). An additional shift was observed in the position of K20 in the first helix (Fig. 2.3D).

Figure 2.3. Structural features of CRABP-II and alignment of holo-CRABP-II-K20/R29/ K30 with the NLS of the SV40-T antigen.. (A) Superposition of the of apo- (blue) (Chen et al., 1998) and holo-CRABP-II (green) (Kleywegt et al., 1994). Bound RA is shown in gray. (B) Computed electrostatic surface potentials of apo- and holo-CRABP-II (see Experimental Procedures). Basic, acidic, and neutral charges are denoted by blue, red, and white, respectively. A positively charged patch (arrow) is manifested in holo-CRABP-II. (C) Structures of helix 2 of apo- and holo-CRABP-II showing the region that tightens in response to RA binding (arrows). (D) Structures of the helix-loop-helix regions of apo- and holo-CRABP-II showing that three basic residues, K20, R29 and K30, change positions upon ligation. (E) Structures of helix 1 showing increased distance between E17 and K20 in the holo-protein. (F) Structure of the SV40 NLS peptide bound to karyopherinα (Conti et al., 1998). K128, K129 and K131 (blue) comprise the consensus NLS of SV40. (G) Superposition of residues K20, R29 and K30 of holo-CRABP-II (green) with K128, K129 and K131 of the SV40 NLS peptide (blue).


A
B
Apo
holo
C
23
23
40
40
apo
holo
D
K30
K20
R29
K20
R29
C
C
apo
holo
E
K20
E17
14
E17
K20
14
apo
holo
F
K129
K131
K128
G
R29
K20
K30

In apo-CRABP-II, K20 is placed in close proximity to E17, suggesting electrostatic interactions between the two residues (Fig. 2.3E). In the holo-protein, a twist in E17 distances it from K20 likely allowing for the observed re-positioning of the latter. Overall, the model shows that all three basic residues of the helix-loop-helix region, K20, R29, and K30, contribute to the basic surface patch that emerges upon protein ligation.

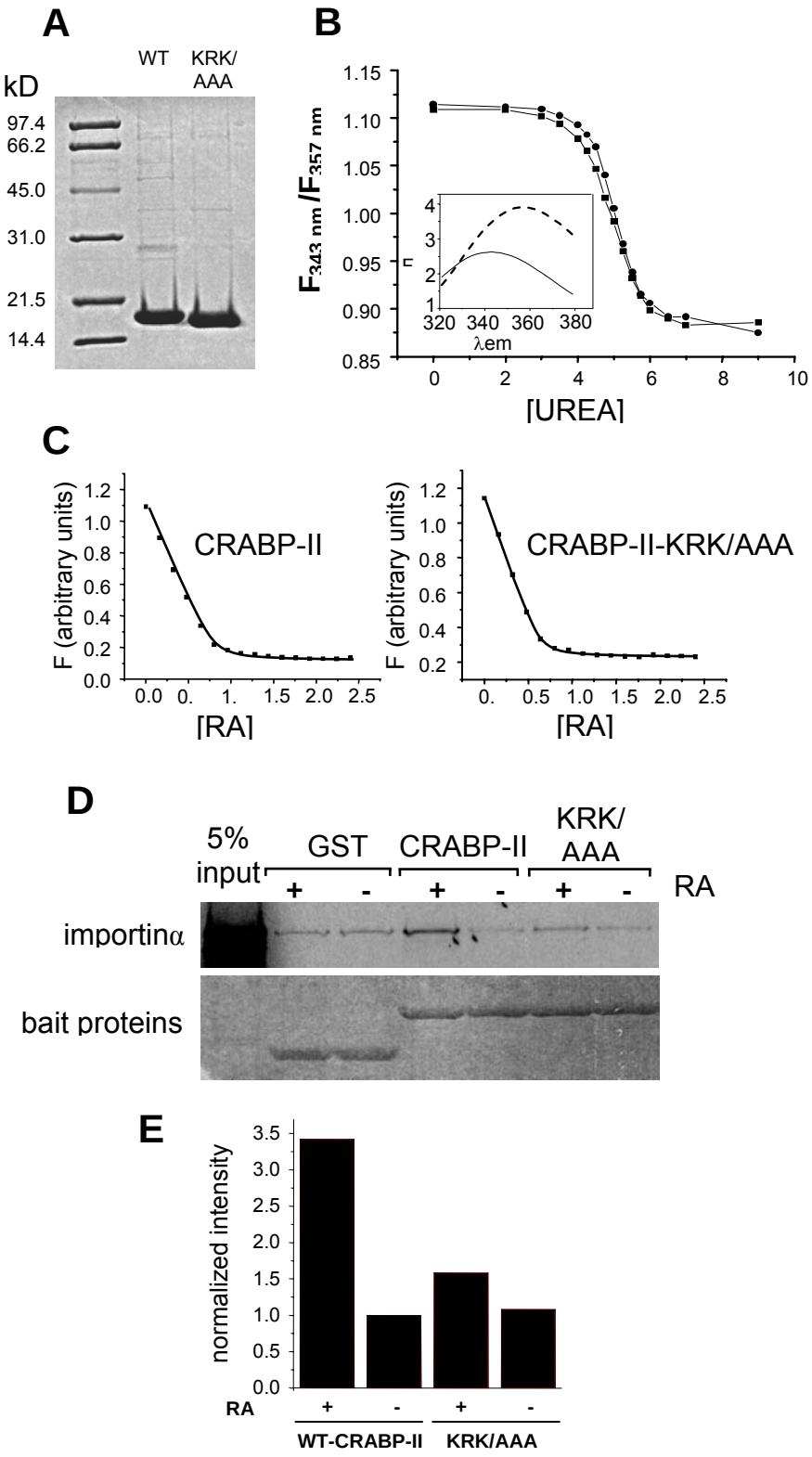
As known NLS sequences are comprised of basic residues, it is possible that the positively charged surface patch of holo-CRABP-II represents a novel NLS. We thus compared the structure of the helix-loop-helix region of holo-CRABP-II to that of the 'classical' NLS of the SV40-T antigen which has been solved in complex with karyopherinα, the yeast orthologue of mammalian importinα (Conti et al., 1998). In the crystal, an SV40 NLS peptide is bound to each of the two NLS binding sites of karyopherinα. One of these sites, known as the "major site", is thought to be more important in NLS recognition (Chook and Blobel, 2001; Conti et al., 1998; Hodel et al., 2001) and was thus used in the analysis (Fig. 2.3F). Comparison of the helix-loop-helix region of holo-CRABP-II with the karyopherinα -bound SV40 NLS peptide (Figure 2.3G) shows a close alignment of CRABP-II-K20, R29, and K30 with the basic residues of the SV40 NLS-K131, K129, and K128, respectively.

2.3.4 RA induces association of CRABP-II with importin α and mutations of K20, R29, and K30A hinder the interactions.

The similarity between the 3-dimensional configuration of the basic helix-loop-helix residues of holo-CRABP-II and a 'classical' NLS suggests that these residues may enable an importin-mediated nuclear transport of the

protein. We thus studied the ability of CRABP-II to bind to an importin and the role of K20, R29, and K30 in such an interaction. A CRABP-II mutant in which these residues were replaced by alanines was generated. To examine whether the mutations disrupt the overall folding of the protein, WT and mutant CRABP-II were expressed in *E. coli*, purified (Fig. 2.4A), and subjected to urea-induced denaturation. Denaturation was followed by monitoring the protein's fluorescence emission spectrum whose peak shifted from 343 nm in the absence of urea to 357 nm upon complete unfolding (Fig. 2.4B, inset). The shifts in the emission spectra of WT-CRABP-II and its K20A/R29A/K30A mutant at increasing urea concentrations were recorded in the form of the ratio of fluorescence intensities at peak emission of the folded and unfolded proteins (Fig. 2.4B). The data show that the urea sensitivities of WT and mutant CRABP-II are all but identical, indicating that the global folding characteristics of the mutant are intact. To further verify the viability of the mutant, the equilibrium dissociation constant (Kd) for its association with RA was measured. Measurements were carried out by fluorescence titrations, a frequently used method based on the observations that, due to the extensive overlap of fluorescence emission spectra of proteins and the absorption spectrum of retinoids. To further verify the viability of the mutant, the equilibrium dissociation constant (Kd) for its association with RA was measured. Measurements were carried out by fluorescence titrations, a frequently used method based on the intrinsic fluorescence of retinoid-binding proteins is quenched upon ligand-binding (Cogan et al., 1976; Fiorella and Napoli, 1991; Kersten et al., 1995; Noy et al., 1992).

Figure 2.4. RA induces association of CRABP-II with importinα and mutations of K20, R29, and K30 hinder the interactions. (A) SDS-PAGE of bacterially expressed WT-CRABP-II and its mutant (KRK/AAA). (B) Urea-induced unfolding of CRABP-II (squares) and mutant (circles). Inset: Fluorescence emission spectra of WT-CRABP-II (λ_{ex} = 283 nm) in the absence (solid line) or presence (dotted line) of 9 M urea. Urea-induced shift in emission spectra is presented as the ratio of fluorescence intensities at 343 nm and 357 nm. (C) Representative fluorescence titrations of WT- and mutant CRABP-II. Data were fitted to an equation derived from simple binding theory (solid line through data points). (D) GST pull-down experiments using ^{35}S-labeled importin hSRP1 and GST, or GST-tagged CRABP-II, or its mutant (KRK/AAA), in the presence or absence of RA (1.5 M). ^{35}S- importin and bait proteins were visualized by autoradiography and by Coomassie-blue staining, respectively. The experiment was carried out three times with similar results. (E) Quantitation of bands in panel 4D. Intensities were measured using Alpha Innotech AlphaEase ver. 5.5 software for image capture and densitometry, and normalized to intensity observed with unliganded WT-CRABP-II.


A
WT
KRK/
AAA
kD
97.4
66.2
45.0
31.0
21.5
14.4
B
F343 nm /F357 nm
[UREA]
λem
C
CRABP-II
CRABP-II-KRK/AAA
F (arbitrary units)
[RA]
D
5%
input
GST
CRABP-II
KRK/
AAA
RA
importinα
bait proteins
E
normalized intensity
RA
WT-CRABP-II
KRK/AAA

It should be noted that, because of sensitivity limitations, fluorescence titrations are usually carried out using protein concentrations that are significantly higher than the Kd (Dong et al., 1999). Hence, derived values should be considered to comprise upper limits for the actual values (see (Noy, 1998)). Analyses of titration curves of WT and mutant CRABP-II (Fig. 2.4C) yielded Kd values of 11.4±8.6 nM and 21.5±10.5 nM, respectively, demonstrating that the mutations did not hamper high affinity RA-binding by the protein.

The ability of CRABP-II to associate with importin α (hSRP1 alpha, (Weis et al., 1995)) was then examined. ^{35}S-labeled importin α was generated by coupled *in vitro* transcription/translation, and GST-tagged CRABPs were purified from over-expressing *E. coli*. Association of importin α with CRABP-II and its K20A/R29A/K30A mutant was studied by co-precipitation assays. GST-tagged CRABPs and GST alone, serving as a control, were immobilized on glutathione agarose beads, and incubated with ^{35}S-importin α in the presence or absence of RA. Beads were centrifuged and washed, and pelleted proteins resolved by SDS-PAGE. Importin α that co-precipitated with immobilized proteins was visualized by autoradiography (Fig. 2.4D). A weak and seemingly non-specific association between CRABP-II and importinα was observed in the absence of ligand, but the interaction was considerably stabilized in the presence of RA. In contrast, association of importinα with the CRABP-II homolog CRABP-I remained at baseline both in the absence and in the presence of RA (data not shown). Mutation of the residues proposed to comprise the NLS of CRABP-II markedly hindered the ligand-stimulated interactions of importinα with the protein. We note that only a small fraction of the importinα input co-precipitated with CRABP-II, suggesting that the

interaction is relatively weak and thus that another importin subtype may be involved in mediating the nuclear import of the protein *in vivo*. Nevertheless, the observations that CRABP-II binds to an importinα, that the interaction is ligand-dependent, and that mutation of K20, R29, and K30 hinders the association strongly support the identification of these residues as the NLS of CRABP-II.

2.3.5 Residues K20, R29, and K30 are critical for ligand-induced nuclear localization of CRABP-II.

The consequences of mutating the putative NLS for the ability of CRABP-II to undergo a ligand-induced nuclear localization were then investigated. COS-7 cells were transfected with expression vectors encoding GFP-tagged WT- or mutant-CRABP-II and imaged prior to or following a 30 min. treatment with RA. The images (Fig. 2.5) clearly show that, in contrast with the WT protein, the sub-cellular distribution of GFP-CRABP-II-K20A/R29A/K30A did not shift upon addition of RA. Hence, residues K20, R29 and K30 are essential for RA-induced nuclear import of CRABP-II.

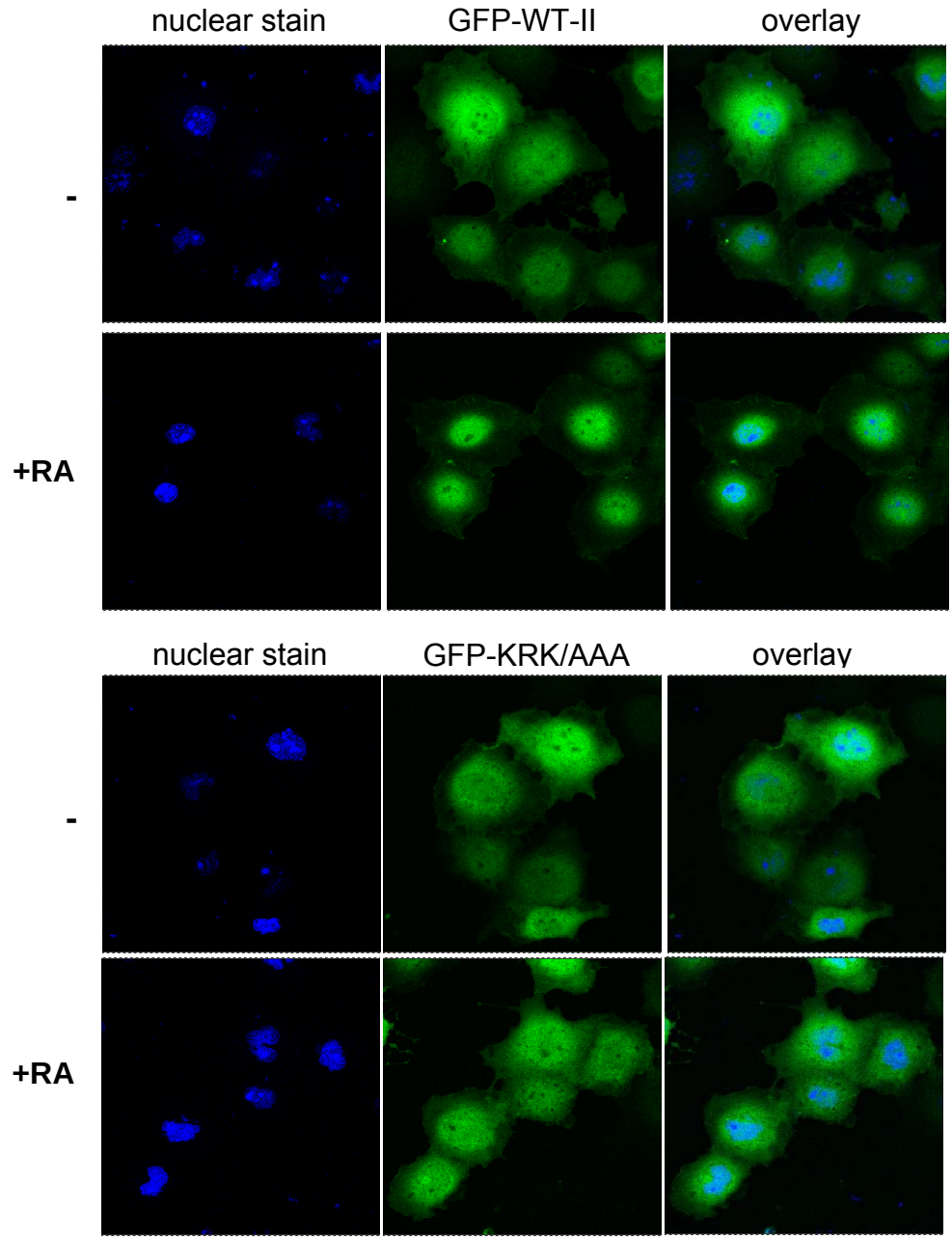


Figure 2.5. Mutation of the putative NLS abolishes ligand-induced nuclear localization of CRABP-II. COS-7 cells were transfected with GFP-WT-CRABP-II or its K20A/R29A/K30A mutant (KRK/AAA). Images of GFP-tagged proteins (green) and of the nuclear marker Red1-nuc (blue) were acquired from live cells before or after a 30 min. treatment with RA (1 μM).

2.3.6 NLS-mediated nuclear localization is critical for enabling CRABP-II to enhance the transcriptional activity of RAR.

To examine whether nuclear localization of CRABP-II is required for the ability of the protein to enhance the transcriptional activity of RAR, we used CRABP-II that harbors an NES and is excluded from the nucleus both in the absence and in the presence of RA (Fig. 2.2). The effects of nuclear exclusion on the transcriptional activity of CRABP-II were studies by transactivation assays. COS-7 cells were transfected with an expression vector harboring WT or NES-CRABP-II together with a luciferase reporter vector driven by an RAR response element. Cells were treated with RA, and luciferase activity was measured (Fig. 2.6). While expression of WT-CRABP-II significantly enhanced the RA-induced, RAR-mediated, activation of the reporter gene, fusing an NES to CRABP-II completely abolished the effect of the binding protein. Hence, movement into the nucleus is essential for the ability of CRABP-II to augment the transcriptional activity of RAR. These observations provide an additional approach for testing whether residues K20, R29, and K30 comprise the NLS of CRABP-II. If these residues indeed mediate the nuclear localization of the protein, their mutation will inhibit the activation of RAR by CRABP-II. To examine this prediction, transactivation assays were carried out in COS-7 cells transfected with CRABP-II-K20A/R29A/K30.

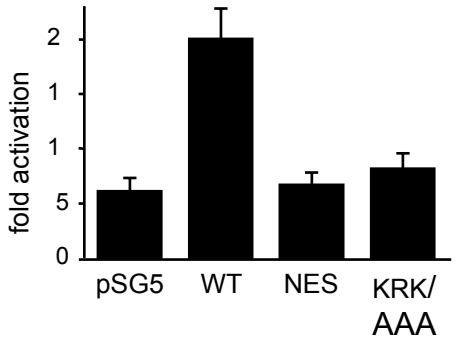


Figure 2.6. Nuclear localization is required for CRABP-II-mediated enhancement of the transcriptional activity of RAR. COS-7 cells were transfected with a luciferase reporter driven by an RAR response element, a pCH110 vector (internal standard) and either an empty vector (pSG5), or expression vectors for the denoted CRABP-II constructs. Cells were treated with RA (250 nM) or vehicle overnight prior to measurements of luciferase activity. Luciferase activity was normalized to β-gal activity and fold activation is presented. Data are means ± standard deviation (*n* = 9).

The data (Fig. 2.6) show that, mutation of the putative NLS dramatically diminished the ability of CRABP-II to augment transcriptional activation by RAR although it did not abolish it completely. The residual activity may be attributed to leakage of the over-expressed mutant into the nucleus even in the absence of a specific NLS (Fig. 2.5).

2.4 DISCUSSION

Many proteins that do not contain a recognizable NLS in their primary sequence are nevertheless found in the nucleus, raising the question of how their nuclear localization is accomplished (Chkheidze and Liebhaber, 2003; Wang et al., 1997; Wolff et al., 2002; Yamaki et al., 2004). The present work was undertaken in order to identify the region in CRABP-II, a member of the iLBP protein family, that mediates its nuclear localization. The question is of particular interest not only because CRABP-II does not harbor a familiar NLS, but also because its NLS functions as a ligand-activated switch triggered by RA (Fig. 2.1). Analyses of the 3-dimensional structures of apo- and holo-CRABP-II revealed a surface patch that is neutral in the absence of ligand and becomes positively charged in the holo-protein. This patch was mapped to three basic amino acid residues, K20, R29, and K30, that shift their orientation upon ligation to place their side chains in a close alignment with the ‘classical’ NLS of the SV40-T antigen when bound to karyopherinα (Fig. 2.3). Hence, subtle, ligand-induced, shifts in the tertiary structure of CRABP-II resulted in the appearance of a recognizable NLS. Subsequent analyses demonstrated that RA induces the association of CRABP-II with importinα, and that mutation of the putative NLS markedly diminishes the ligand-stimulated interaction (Fig.

2.4D and 2.4E). Correspondingly, we found that K20, R29, and K30 are critical for mediating the nuclear localization of CRABP-II (Fig. 2.5) and for enabling the protein to exert its nuclear activity (Fig. 2.6). Taken together, the data substantiate that K20, R29, and K30 of CRABP-II function as the protein's NLS. The identification of this signal suggests that proteins that do not possess a nuclear targeting sequence in their primary structure may localize to the nucleus by using an NLS which is manifested in their folded state. It is interesting to note that nuclear import of STAT1 and STAT2 was reported to be mediated by a related phenomenon. Specifically, it was shown that interferon-stimulated phosphorylation of these proteins induces their heterodimerization and creates a 3-dimentional arrangement of a Lys/Arg-rich region which forms a functional NLS (Fagerlund et al., 2002; Melen et al., 2001). Taken together with the present data, these observations suggest that "structural NLSs" may function to enable the regulation of the subcellular distribution of proteins by post-translational mechanisms.

Proteins that harbor classical NLSs are often targeted to the nucleus through association with α-importins (Chook and Blobel, 2001). Despite the tight sequence conservation of such NLS, binding affinities of importins towards their multiple cargoes differ widely (Freedman and Yamamoto, 2004; Kohler et al., 1999; Miyamoto et al., 1997). Hence, although the presence of an NLS is critical for nuclear localization, association with the appropriate importin appears to be regulated by other features, as for example, by the exact nature of the regions flanking the NLS. The present findings demonstrate that CRABP-II possesses a version of a classical NLS which enables its importin-mediated nuclear transport. The identity of the importin isotype that mediates the process *in vivo*, and the additional structural

characteristics that contribute to importin-binding by CRABP-II remain to be elucidated.

In the nucleus, CRABP-II directly interacts with the nuclear receptor RAR to form a complex that mediates RA-channeling to the receptor. Consequently, CRABP-II facilitates the ligation of RAR, augments its transcriptional activity, and dramatically enhances the sensitivity of cells to the biological activities of RA (Budhu et al., 2001; Budhu and Noy, 2002; Dong et al., 1999; Manor et al., 2003). Similarly to CRABP-II, two other iLBPs, A-FABP and K-FABP, were shown to move to the nucleus in response to cognate ligands and to enhance the transcriptional activities of particular nuclear receptors (Tan et al., 2002). Also similarly to CRABP-II, neither of these iLBPs harbor a recognizable NLS in their primary sequence. Alignment of the primary sequences of several iLBPs (Fig. 2.7) shows that the three basic residues that comprise the NLS of CRABP-II are present in both A-FABP and K-FABP, suggesting that they also may possess a CRABP-II-like “3-dimensional NLS”.

The alignment of the primary sequences of iLBPs (Fig. 2.7) further shows that residues homologous to the CRABP-II NLS are present in proteins that are believed to function outside the nucleus, i.e. CRABP-I, CRBP-I, and CRBP-II. These observations suggest that the configuration of these residues is distinct in different iLBPs.

```
FABP1(lFABP) ....MSFSGK YQLQSQENFE AFMKAIGLPE ELIQ..KGKD IKGVSEIVQN
FABP2(iFABP) ....MAFDST WKVDRSENYD KFMEKMGVNI VKRK..LAAH DNLKLTITQE
FABP3(hFABP) ..MVDAFLGT WKLVDSKNFD DYMKSLGVGF ATRQ..VASM TKPTTIIEKN
FABP4(aFABP) ..MCDAFVGT WKLVSSENFD DYMKEVGVGF ATRK..VAGM AKPNMIISVN
FABP5(eFABP) MATVQQLEGR WRLVDSKGFD EYMKELGVGI ALRK..MGAM AKPDCIITCD
CRABP-I      ...MPNFAGT WKMRSSENFD ELLKALGVNA MLRKVAVAAA SKPHVEIRQD
CRABP-II     ...MPNFSGN WKIIRSENFE ELLKVLGVNV MLRKIAVAAA SKPAVEIKQE
CRBP-I       ..MPVDFTGY WKMLVNENFE EYLRALDVNV ALRK..IANL LKPDKEIVQD
CRBP-II      ..MTRDQNGT WEMESNENFE GYMKALDIDF ATRK..IAVR LTQTKVIDQD
```

Figure 2.7. Alignment of iLBPs with the NLS-containing region of CRABP-II. Some iLBPs contain residues that correspond in charge to the CRABP-II-NLS (underlined), while others do not (bold). Alignment was accomplished using accession #s: NP_001869 (hCRABP-II), P15090 (hA-FABP), NP_001435 (hK-FABP), NP_004369 (hCRABP-I), NP_002890 (hCRBP-I), NP_004155 (hCRBP-II), NP_001434 (hL-FABP), P12104 (hI-FABP), and P05413 (hH-FABP).

Indeed, it was reported that ligand-binding by various iLBPs alters the structures of their helix-loop-helix regions, (Hodsdon and Cistola, 1997; Wang and Yan, 1999), but that the resulting configuration and structural dynamics are distinct. For example, in CRBP-I, the region appears to be loose in the apo-protein and to become more rigid upon binding of retinol. In contrast, the corresponding region in CRBP-II is rigid both in the apo- and in the holo-forms (Lu et al., 2003). Interestingly, while CRABP-I contains basic residues in positions corresponding to the NLS of CRABP-II, it does not target the nucleus in response to RA (Budhu and Noy, 2002). Preliminary comparison of the structures of CRABP-I and CRABP-II suggests that two of the CRABP-II-NLS residues, K20 and K30, are aligned differently in CRABP-I. Other features of the proteins, such as their dynamics or their overall patterns of hydrophobicity and electrostatic potential, may also contribute to the differential subcellular location of the two CRABPs.

The diversity and evolutionary conservation of members of the iLBP family indicate that they play important roles in the biology of their ligands, but, to-date, neither sequence analyses nor structural data have provided clear insights into their functions. Here we show that the helix-loop-helix of iLBPs can function as a switch that is controlled by ligand-induced subtle but concerted rearrangements of multiple protein regions. In the case of CRABP-II, and perhaps K-FABP and A-FABP, the region functions as an NLS. Considering the structural similarities of iLBPs, it is likely that the ability of the region to relay ligand-binding information is common throughout the family. This switch may serve to direct different iLBPs to different cellular targets, enabling them to mediate divergent functions in response to their cognate ligands.

2.5 EXPERIMENTAL PROCEEDURES

2.5.1 Reagents

Antibodies for CRABP-II were provided by Pierre Chambon (IGBMC, Illkirch, France). RA was purchased from Sigma Chemical Co.

2.5.2 Modeling

Modeling was carried out using coordinates from 1XCA (Chen et al., 1998), 1CBS and 1CBR (Kleywegt et al., 1994), and 1BK6 (Conti et al., 1998) deposited in the Research Collaboratory for Structural Bioinformatics (RCSB) protein database (http://www.rcsb.org/pdb/) for apo-CRABP-II, holo-CRABP-II, holo-CRABP-I, and Karyopherinα bound to the SV40 NLS peptide, respectively. In the crystals, both CRABPs and karyopherinα are dimeric. In all cases, the coordinates for the first monomer were used. The available X-ray crystal structure for apo-CRABP-II is of a mutant (R111A) (Chen et al., 1998). The mutation however is distant from the region studied and is believed to cause minimal perturbation of overall protein fold . Molecular surfaces and electrostatic potentials were calculated using Swiss-PDB viewer ver. 3.6b3 (http://www.expasy.org/spdbv/) (Guex and Peitsch, 1997). Swiss-PDB viewer images were saved as pov3.1 files and rendered using POVRAY for windows, version 3.5 (http://www.povray.org/). Sequence alignment was done using the *Multalin* multiple sequence alignment program (http://prodes.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1988).

2.5.3 Vectors

Mammalian expression vectors for hCRABP-II in pEGFP-C2, hCRABP-I in pSG5, hCRABP-II in pSG5, and bacterial expression vector for hCRABP-II in pET28a were previously described (Budhu et al., 2001; Budhu and Noy, 2002). Expression vector for importinα (hSRP1) was a gift from Karsten Weis (University of California, Berkeley).

To generate NES-CRABP-II in pcDNA3.1, CRABP-II cDNA containing restriction sites for *EcoRI* (5') and *Xho1* (3') was created by PCR amplification using hCRABP-II-pSG5 as a template. Using *EcoR1* and *Xho1*, K-FABP was cut from the K-FABP-NES-pcDNA3.1 vector (Tan et al., 2002) and replaced by the CRABP-II PCR product. GFP-NES-CRABP was generated by cloning NES-CRABP-II cDNA into pEGFP-C2 (Clontech) using restriction sites for *Kpn1* (5') and *BamH1* (3'). GST-CRABP-II and GST-CRABP-I were generated by cloning the respective cDNAs into pGEX-4T2 (Amersham) using restriction sites for *BamH1* (5') and *EcoR1* (3'). CRABP-II-K20A/R29A/K30A was generated using the Stratagene quikchange site-directed mutagenesis kit. Template vectors were used to generate hCRABP-II-K20A, and the resultant vectors were used to create CRABP-II-K20A/R29A/K30A.

2.5.4 Bacterially expressed proteins

GST-tagged proteins were expressed in the *E. coli* strain BL21. Bacteria were grown in LB medium at 37°C to an $O.D._{600\ nm}$ of 0.7, and protein expression induced by 0.5 mM IPTG. Following an additional 3 hr. at 30°C, bacteria were centrifuged and frozen at –20°C until use. Pellets were homogenized in buffer A (500 mM NaCl, 20 mM TRIS, pH 7.4, 5 mM imidazole, 40 ml/1 l culture) containing lysozyme and PMSF (200 µM).

Lysates were incubated with stirring (4°C, 20 min.), sonicated, centrifuged, and supernatants applied to a nickel-charged imidodiacetic acid agarose column (1 ml/1 l culture). Column was rocked (3 hr., 4°C), washed with 3 volumes buffer A containing 50 mM imidazole, and protein eluted with buffer A containing 500 mM imidazole (10 ml/elution, 3 times). Eluates were pooled, dialyzed against HEDK buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM DTT , 100 mM KCl), concentrated, diluted 1:2 with glycerol, and stored at –20°C. GST-tagged proteins were expressed and purified essentially the same way using HEDK buffer and glutathione-agarose. Proteins were eluted (20 mM tris, pH 8.0, 10 mM glutathione), dialyzed against HEDK, concentrated, diluted 1:2 with glycerol and stored at –20°C. Protein concentrations were measured by the Bradford assay ((Bradford, 1976), BioRad), and viability assessed by monitoring RA-binding using fluorescence titrations.

2.5.5 Urea-induced unfolding

Proteins (1 μM) were incubated at varying urea concentrations (37ºC, 15 min). Emission spectra were recorded at 37ºC using a SPEX Industries Fluorolog 2 spectrofluorometer.

2.5.6 Fluorescence titrations

Protein (1 μM) was place in a cuvette and titrated with RA from a concentrated solution in ethanol. Titrations were monitored by following the ligand-induced decrease in the intrinsic fluorescence of the protein (λ_{ex} -280 nm; λ_{em} -340 nm).

2.5.7 Coprecipitation assays

^{35}S-labeled importinα was generated by coupled *in vitro* transcription/translation (T7-TnT Coupled Reticulocyte Lysate System, Promega) following the manufacturer's protocol.. Pull-down mixtures (1 ml in 50 mM HEPES, pH 8.0, 100 mM NaCl, 0.05% NP40, 0.1% BSA) contained 650 pmoles GST-tagged protein, 100 µl glutathione agarose (1/10 slurry), 50 µl of TnT reaction, and RA (1.5 µM) or vehicle (ethanol). Mixtures were incubated (4°C, 2 hr) and centrifuged. Pelleted beads were washed (800 µl buffer containing RA or vehicle, 4 times), resuspended in 30 µl 2.5x SDS-PAGE loading buffer, boiled, and resolved by 10% SDS-PAGE. Gels were stained by Coomassie-blue, dried, and autoradiographed.

2.5.8 Fluorescence microscopy

To image endogenous CRABP-II, MCF-7 cells were seeded on multi-well glass chamber slides and grown to 50-75% confluence in DMEM supplemented with 5% charcoal-treated FBS. Medium was replaced by serum-free DMEM, and cells grown overnight followed by treatment with RA (100 nM, 30 min.) or vehicle.

Cells were fixed using 3.7% formaldehyde in PBS (RT, 10 min.), permeabilized with Triton X-100 (0.2 % in PBS, 37°C, 10 min.), washed, and incubated with mouse monoclonal anti-CRABP-II antibodies (1:250 in PBS containing 2% BSA, 1hr.). Cells were then incubated with the nucleic acid stain ToPro3 (Molecular Probes, 4 µl/ml in PBS, 15 min.), and with rabbit anti-mouse Alexafluor-conjugated antibodies (Molecular Probes, 1:500 in PBS containing 2% BSA, 1 hr.), mounted with coverslips using *slowfade light*

reagent (Molecular Probes) and imaged using a Leica TCS SP2 confocal microscope system.

To image GFP-tagged proteins, COS-7 cells were plated in 35 mm glass bottom microwell dishes (Mattek) in DMEM containing 5% charcoal-treated FBS (75,000 cells per plate) and grown overnight. Cells were transfected using Fugene (Roche) with an expression vector harboring the appropriate CRABP-II construct (250 ng DNA per plate) and pHcRed1-nuc (Clontech, 200 ng per plate). Following an overnight incubation, medium was replaced with serum-free DMEM, and live cells were imaged using a Leica TCS SP2 confocal microscope equipped with a 40x dipping lens. Fluorescence of GFP and Red1-nuc were collected simultaneously. After imaging, cells were treated with 1 μM RA, incubated at 37°C for 30 min. and imaged again.

2.5.9 Transactivation assays

COS-7 cells were seeded in 12 well plates (75,000 cells per well) in 10% FBS in DMEM, grown overnight, and transfected with 500 ng RARE-luc reporter vector, 100 ng pCH110, and 500 ng of test plasmid. Following an overnight incubation, medium was replaced with serum-free DMEM and cells treated with 250 nM RA or vehicle overnight. Cell lysates were assayed for luciferase activity using the Luciferase Assay System (Promega). Luciferase activity was corrected for transfection efficiency by the activity of β-galactosidase.

2.6 ACKNOWLEDGEMENTS

We are grateful to Richard Gillilan (Cornell University) for helpful discussions, Pierre Chambon (IGBMC, Illkirch, France) for CRABP-II constructs and antibodies, and Karsten Weis (University of California, Berkeley) for importin□/hSRP1 expression vector. This work was supported by NIH grants R01-DK60684 and R01-CA68150. RJS was supported by NIH grant T32-CA09682.

2.7 REFERENCES

Aranda, A., and Pascual, A. (2001). Nuclear hormone receptors and gene expression. Physiol Rev *81*, 1269-1304.

Bastien, J., and Rochette-Egly, C. (2004). Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene *328*, 1-16.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*, 248-254.

Budhu, A., Gillilan, R., and Noy, N. (2001). Localization of the RAR interaction domain of cellular retinoic acid binding protein-II. J Mol Biol *305*, 939-949.

Budhu, A. S., and Noy, N. (2002). Direct channeling of retinoic acid between cellular retinoic acid-binding protein II and retinoic acid receptor sensitizes mammary carcinoma cells to retinoic acid-induced growth arrest. Mol Cell Biol *22*, 2632-2641.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. Faseb J *10*, 940-954.

Chen, X., Tordova, M., Gilliland, G. L., Wang, L., Li, Y., Yan, H., and Ji, X. (1998). Crystal structure of apo-cellular retinoic acid-binding protein type II (R111M) suggests a mechanism of ligand entry. J Mol Biol *278*, 641-653.

Chkheidze, A. N., and Liebhaber, S. A. (2003). A novel set of nuclear localization signals determine distributions of the alphaCP RNA-binding proteins. Mol Cell Biol *23*, 8405-8415.

Chook, Y. M., and Blobel, G. (2001). Karyopherins and nuclear import. Curr Opin Struct Biol *11*, 703-715.

Cogan, U., Kopelman, M., Mokady, S., and Shinitzky, M. (1976). Binding affinities of retinol and related compounds to retinol binding proteins. Eur J Biochem *65*, 71-78.

Conti, E., Uy, M., Leighton, L., Blobel, G., and Kuriyan, J. (1998). Crystallographic analysis of the recognition of a nuclear localization signal by the nuclear import factor karyopherin alpha. Cell *94*, 193-204.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res *16*, 10881-10890.

Dalman, F. C., Sturzenbecker, L. J., Levin, A. A., Lucas, D. A., Perdew, G. H., Petkovitch, M., Chambon, P., Grippo, J. F., and Pratt, W. B. (1991). Retinoic acid receptor belongs to a subclass of nuclear receptors that do not form "docking" complexes with hsp90. Biochemistry *30*, 5605-5608.

Desvergne, B., and Wahli, W. (1999). Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev *20*, 649-688.

Dilworth, F. J., and Chambon, P. (2001). Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. Oncogene *20*, 3047-3054.

Dingwall, C., and Laskey, R. A. (1991). Nuclear targeting sequences--a consensus? Trends Biochem Sci *16*, 478-481.

Dong, D., Ruuska, S. E., Levinthal, D. J., and Noy, N. (1999). Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. J Biol Chem *274*, 23695-23698.

Fagerlund, R., Melen, K., Kinnunen, L., and Julkunen, I. (2002). Arginine/Lysine-rich localization signals mediate interactions between dimeric STATs and importin alpha5. J Biol Chem *277*, 30072-30078.

Fiorella, P. D., and Napoli, J. L. (1991). Expression of cellular retinoic acid binding protein (CRABP) in Escherichia coli. Characterization and evidence that holo-CRABP is a substrate in retinoic acid metabolism. J Biol Chem *266*, 16572-16579.

Forman, B. M., Chen, J., and Evans, R. M. (1997). Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A *94*, 4312-4317.

Freedman, N. D., and Yamamoto, K. R. (2004). Importin 7 and importin alpha/importin beta are nuclear import receptors for the glucocorticoid receptor. Mol Biol Cell *15*, 2276-2286.

Guex, N., and Peitsch, M. C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis *18*, 2714-2723.

Gutierrez-Gonzalez, L. H., Ludwig, C., Hohoff, C., Rademacher, M., Hanhoff, T., Ruterjans, H., Spener, F., and Lucke, C. (2002). Solution structure and backbone dynamics of human epidermal-type fatty acid-binding protein (E-FABP). Biochem J *364*, 725-737.

Hagens, G., Masouye, I., Augsburger, E., Hotz, R., Saurat, J. H., and Siegenthaler, G. (1999a). Calcium-binding protein S100A7 and epidermal-type fatty acid-binding protein are associated in the cytosol of human keratinocytes. Biochem J *339 (Pt 2)*, 419-427.

Hagens, G., Roulin, K., Hotz, R., Saurat, J. H., Hellman, U., and Siegenthaler, G. (1999b). Probable interaction between S100A7 and E-FABP in the cytosol of human keratinocytes from psoriatic scales. Mol Cell Biochem *192*, 123-128.

Hanhoff, T., Lucke, C., and Spener, F. (2002). Insights into binding of fatty acids by fatty acid binding proteins. Mol Cell Biochem *239*, 45-54.

Hodel, M. R., Corbett, A. H., and Hodel, A. E. (2001). Dissection of a nuclear localization signal. J Biol Chem *276*, 1317-1325.

Hodsdon, M. E., and Cistola, D. P. (1997). Discrete backbone disorder in the nuclear magnetic resonance structure of apo intestinal fatty acid-binding protein: implications for the mechanism of ligand entry. Biochemistry *36*, 1450-1460.

Jenkins-Kruchten, A. E., Bennaars-Eiden, A., Ross, J. R., Shen, W. J., Kraemer, F. B., and Bernlohr, D. A. (2003). Fatty acid-binding protein-hormone-sensitive lipase interaction. Fatty acid dependence on binding. J Biol Chem *278*, 47636-47643.

Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984). A short amino acid sequence able to specify nuclear location. Cell *39*, 499-509.

Kersten, S., Pan, L., Chambon, P., Gronemeyer, H., and Noy, N. (1995). Role of ligand in retinoid signaling. 9-cis-retinoic acid modulates the oligomeric state of the retinoid X receptor. Biochemistry *34*, 13717-13721.

Kim, H. K., and Storch, J. (1992). Mechanism of free fatty acid transfer from rat heart fatty acid-binding protein to phospholipid membranes. Evidence for a collisional process. J Biol Chem *267*, 20051-20056.

Kleywegt, G. J., Bergfors, T., Senn, H., Le Motte, P., Gsell, B., Shudo, K., and Jones, T. A. (1994). Crystal structures of cellular retinoic acid binding proteins I and II in complex with all-trans-retinoic acid and a synthetic retinoid. Structure *2*, 1241-1258.

Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., and Lehmann, J. M. (1997). Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci U S A *94*, 4318-4323.

Kohler, M., Speck, C., Christiansen, M., Bischoff, F. R., Prehn, S., Haller, H., Gorlich, D., and Hartmann, E. (1999). Evidence for distinct substrate specificities of importin alpha family members in nuclear protein import. Mol Cell Biol *19*, 7782-7791.

Laudet, V., and Gronemeyer, H. (2002). The NuclearReceptor FactsBook (London and San Diego, Academic Press).

Levin, M. S., Locke, B., Yang, N. C., Li, E., and Gordon, J. I. (1988). Comparison of the ligand binding properties of two homologous rat apocellular retinol-binding proteins expressed in Escherichia coli. J Biol Chem *263*, 17715-17723.

Lu, J., Cistola, D. P., and Li, E. (2003). Two homologous rat cellular retinol-binding proteins differ in local conformational flexibility. J Mol Biol *330*, 799-812.

Manor, D., Shmidt, E. N., Budhu, A., Flesken-Nikitin, A., Zgola, M., Page, R., Nikitin, A. Y., and Noy, N. (2003). Mammary carcinoma suppression by cellular retinoic acid binding protein-II. Cancer Res *63*, 4426-4433.

Melen, K., Kinnunen, L., and Julkunen, I. (2001). Arginine/Lysine-rich structural element is involved in interferon-induced nuclear import of STATs. J Biol Chem *276*, 16447-16455.

Miyamoto, Y., Imamoto, N., Sekimoto, T., Tachibana, T., Seki, T., Tada, S., Enomoto, T., and Yoneda, Y. (1997). Differential modes of nuclear localization signal (NLS) recognition by three distinct classes of NLS receptors. J Biol Chem *272*, 26375-26381.

Moroianu, J. (1999). Nuclear import and export: transport factors, mechanisms and regulation. Crit Rev Eukaryot Gene Expr *9*, 89-106.

Nagpal, S., Saunders, M., Kastner, P., Durand, B., Nakshatri, H., and Chambon, P. (1992). Promoter context- and response element-dependent specificity of the transcriptional activation and modulating functions of retinoic acid receptors. Cell *70*, 1007-1019.

Norris, A. W., and Spector, A. A. (2002). Very long chain n-3 and n-6 polyunsaturated fatty acids bind strongly to liver fatty acid-binding protein. J Lipid Res *43*, 646-653.

Noy, N. (1998). Measurement of rates of dissociation of retinoids from the interphotoreceptor retinoid-binding protein. Methods Mol Biol *89*, 177-189.

Noy, N., and Blaner, W. S. (1991). Interactions of retinol with binding proteins: studies with rat cellular retinol-binding protein and with rat retinol-binding protein. Biochemistry *30*, 6380-6386.

Noy, N., Slosberg, E., and Scarlata, S. (1992). Interactions of retinol with binding proteins: studies with retinol-binding protein and with transthyretin. Biochemistry *31*, 11118-11124.

Palmeri, D., and Malim, M. H. (1999). Importin beta can mediate the nuclear impor of an arginine-rich nuclear localization signal in the absence of importin alpha. Mol Cell Biol *19*, 1218-1225.

Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991). Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell *64*, 615-623.

Shen, W. J., Liang, Y., Hong, R., Patel, S., Natu, V., Sridhar, K., Jenkins, A., Bernlohr, D. A., and Kraemer, F. B. (2001). Characterization of the functional

interaction of adipocyte lipid-binding protein with hormone-sensitive lipase. J Biol Chem *276*, 49443-49448.

Sweitzer, T. D., Love, D. C., and Hanover, J. A. (2000). Regulation of nuclear import and export. Curr Top Cell Regul *36*, 77-94.

Tan, N. S., Shaw, N. S., Vinckenbosch, N., Liu, P., Yasmin, R., Desvergne, B., Wahli, W., and Noy, N. (2002). Selective cooperation between fatty acid binding proteins and peroxisome proliferator-activated receptors in regulating transcription. Mol Cell Biol *22*, 5114-5127.

Truant, R., and Cullen, B. R. (1999). The arginine-rich domains present in human immunodeficiency virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. Mol Cell Biol *19*, 1210-1217.

Veerkamp, J. H., and Maatman, R. G. (1995). Cytoplasmic fatty acid-binding proteins: their structure and genes. Prog Lipid Res *34*, 17-52.

Wang, L., and Yan, H. (1999). NMR study of the binding of all-trans-retinoic acid to type II human cellular retinoic acid binding protein. Biochim Biophys Acta *1433*, 240-252.

Wang, P., Palese, P., and O'Neill, R. E. (1997). The NPI-1/NPI-3 (karyopherin alpha) binding site on the influenza a virus nucleoprotein NP is a nonconventional nuclear localization signal. J Virol *71*, 1850-1856.

Weis, K., Mattaj, I. W., and Lamond, A. I. (1995). Identification of hSRP1 alpha as a functional receptor for nuclear localization sequences. Science *268*, 1049-1053.

Widstrom, R. L., Norris, A. W., and Spector, A. A. (2001). Binding of cytochrome P450 monooxygenase and lipoxygenase pathway products by heart fatty acid-binding protein. Biochemistry *40*, 1070-1076.

Wolff, T., Unterstab, G., Heins, G., Richt, J. A., and Kann, M. (2002). Characterization of an unusual importin alpha binding motif in the borna disease virus p10 protein that directs nuclear import. J Biol Chem *277*, 12151-12157.

Wootan, M. G., Bernlohr, D. A., and Storch, J. (1993). Mechanism of fluorescent fatty acid transfer from adipocyte fatty acid binding protein to membranes. Biochemistry *32*, 8622-8627.

Xu, L., Glass, C. K., and Rosenfeld, M. G. (1999). Coactivator and corepressor complexes in nuclear receptor function. Curr Opin Genet Dev *9*, 140-147.

Yamaki, A., Kudoh, J., Shimizu, N., and Shimizu, Y. (2004). A novel nuclear localization signal in the human single-minded proteins SIM1 and SIM2. Biochem Biophys Res Commun *313*, 482-488.