Evidence for a Novel Cardiac-enriched Retinoid X Receptor Partner*

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Recent studies indicate that retinoid-mediated pathways play a pivotal role in cardiac morphogenesis and function. To identify proteins that serve as interacting partners of the retinoid X receptor α (RXRα) in heart, DNA-protein binding studies were performed with an RXR-responsive element (NRRE-1) derived from the medium chain acyl-CoA dehydrogenase gene promoter and nuclear protein extracts prepared from adult rat heart. NRRE-1 is a pleiotropic RXR-responsive element comprised of three potential recognition sites for class II members of the nuclear receptor superfamily. Gel mobility shift assays performed with an NRRE-1 probe in the absence or presence of bacterially overproduced RXRα and nuclear protein extracts prepared from adult rat heart, liver, or brain identified a cardiac-specific, RXR-dependent DNA-protein interaction. The NRRE-1-RXR-cardiac-enriched RXR-interacting protein (CERIP) complex exhibited a distinct mobility compared with NRRE-1-RXR-peroxisome proliferator-activated receptor, NRRE-1-RXR-retinoic acid receptor, or NRRE-1-RXR-thyroid receptor complexes. Mutational analysis demonstrated that two of the three potential binding half-sites of NRRE-1 (an everted repeat separated by an 8-base pair spacer) are required for the NRRE-1-RXR-CERIP interaction. Gel mobility shift assays demonstrated that CERIP interacted with RXRα and RXRγ but not with RXRβ, indicating a receptor subtype-specific binding preference and suggesting an RXR AB region-dependent interaction. The RXR-CERIP complex did not form on NRRE-1 when a mutant GST-RXRα fusion protein lacking the NH2-terminal AB region (but containing the receptor dimerization domain) of RXRα was added in place of the full-length RXRα, confirming a role for the AB region in the RXR-CERIP interaction. DNA-protein cross-linking studies demonstrated that CERIP is a DNA-binding protein of approximately 110 kDa. These results provide evidence for the existence of a cardiac-enriched DNA-binding protein that interacts with RXRα via the AB region and suggest a mechanism whereby cardiac retinoid signaling is controlled in an RXR subtype-specific manner.

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The retinoid X receptor (RXR) is a pleiotropic nuclear receptor transcription factor that interacts with a variety of nuclear receptor dimeric partners. RXR binds cognate response elements as a homodimer in the presence of its ligand, 9-cis retinoic acid, or as a heterodimer with other members of the nuclear hormone receptor superfamily including retinoic acid receptors (RARs), thyroid hormone receptors (TRs), vitamin D receptors, and peroxisome proliferator-activated receptors (PPARs), among others (1–5). In addition to binding DNA as a dimeric partner with other nuclear receptors, RXR interacts with co-activators and co-repressors known to confer transcriptional regulatory properties (6–12). The existence of three distinct RXR subtypes (α, β, and γ) and multiple isoforms generated by alternate promoter utilization and/or differential splicing adds to the complexity of RXR-mediated transcriptional regulatory pathways (13).

Several lines of investigation have indicated that retinoid signaling pathways are involved in cardiac development and function. Offspring of rodents fed a vitamin A-deficient diet display a variety of congenital cardiac defects (14, 15). Human embryos of mothers treated with Accutane, a vitamin A analogue used to treat skin disorders, have a high incidence of heart defects such as transposition of the great vessels, tetralogy of Fallot, and ventricular septal defects (16). The characterization of RXRa null mice has provided insight into the role of RXR in cardiac development and function (17–19). RXRa−/− mice die of heart failure at embryonic day 15 because of a poorly developed ventricular myocardium (18). The incidence of conotruncal defects and other cardiac malformations is high in RXRa mutants (20), particularly in combination with mutations in RARα or RARβ (21). Taken together, these data indicate that retinoid signaling pathways play a critical role in cardiac development.

RXR has also been shown to control the expression of genes involved in postnatal cardiac metabolism and contractile function. We have shown that RXRα activates the transcription of the genes encoding medium chain acyl-CoA dehydrogenase (MCAD); Refs. 22 and 23) and muscle-type carnitine palmitoyl-transferase I (24), two enzymes involved in mitochondrial fatty acid oxidation, a pathway that is critical for postnatal cardiac energy transduction. Studies performed in cell culture (24, 25) and in vivo (26, 27) have demonstrated that basal and fatty acid stimulated expression of the MCAD and muscle carnitine palmitoyltransferase I genes in heart and liver is controlled in part by RXRα/PPARα heterodimers. Previous studies have also
shown that the slow sarcoplasmic reticulum Ca\(^{2+}\) ATPase (28) and cardiac \(\alpha\) and \(\beta\) myosin heavy chain genes (29, 30) are regulated by RXR/thyroid receptor heterodimers. Lastly, two studies have demonstrated that RXR-dependent pathways suppress the cardiac myocyte hypertrophy program (31, 32). Taken together, these studies define an important role for RXR and retinoid signaling in postnatal heart.

Despite the explosion of new information regarding the diverse mechanisms whereby retinoid receptors control gene expression, little is known about the mechanisms involved in cell-, organ-, and developmental stage-specific actions of RXR. Such specificity could be achieved via distinct RXR subtypes/isoforms, heterodimeric partner selection, or ligand availability. The recent identification of a diverse number of RXR-interacting proteins suggests another mechanism for cell- and tissue-specific control of retinoid signaling, through the availability of specific core-activators or co-repressors of RXR. However, the majority of RXR interacting proteins identified to date are ubiquitously expressed and generally do not exhibit receptor specificity. We speculated that one potential mechanism for the cardiac-specific action of RXR is via interactions with cardiac-enriched interacting proteins or DNA-binding partners.

To test this hypothesis, we utilized the known pleiotropic RXR response element identified within the human MCAD gene promoter termed nuclear receptor response element 1 or NRRE-1 (22, 23). NRRE-1 is a complex retinoid-responsive element comprised of three potential hexameric binding sites for class II members of the nuclear hormone superfAMILY (23). The various pairwise combinations of the binding sites within NRRE-1 define at least three separate elements: an everted repeat separated by 8 base pairs (ER8), an imperfect direct repeat (DR0), and an everted repeat separated by 13 base pairs (ER13) (23). Experiments performed in vitro have demonstrated that NRRE-1 is capable of binding alternative pairs of RXR heterodimers including RXR-RAR and RXR-PPAR in vitro (22, 23, 25) but not RXR homodimers. NRRE-1 also interacts with chicken ovalbumin upstream promoter transcription factor I (COP TF I) homodimers and estrogen-related receptor \(\alpha\) homodimers in an RXR-independent manner (23, 23). Murine transgenic experiments have shown that NRRE-1 is necessary for appropriate expression during cardiac perinatal development and in the adult heart (26). Because NRRE-1 is capable of interacting with a variety of RXR heterodimers in vitro and given its importance in the control of metabolic gene expression in heart, it was utilized as a target element for the identification of RXR partners in nuclear protein extracts prepared from adult rat heart. In this report, we describe the identification of a cardiac-specific, RXR-dependent NRRE-1-binding protein. Surprisingly, the interaction of this cardiac enriched RXR-interacting protein (CERIP) with RXR is isoform-specific and requires the amino-terminal AB region of RXR rather than the classical dimerization domain. In addition, based on UV cross-linking studies, CERIP is a DNA-binding protein. These findings identify a potentially novel cardiac-enriched RXR partner, define an important role for the AB domain of this nuclear receptor, and suggest a mechanism for the cardiac- and subtype-specific actions of RXR.

**MATERIALS AND METHODS**

*Electrophoretic Mobility Shift and Antibody Supershift Assays—* Electrophoretic mobility shift assays (EMSA) were performed as described previously (34) using the normal and mutant NRRE-1 double-stranded oligonucleotide probes described in Fig. 3A. A double-stranded oligonucleotide probe derived from the nuclear receptor response element in the mouse MCAD gene promoter (35) was used for the EMSA shown in Fig. 4 (sense strand sequence, 5'-gctatcatagacagtctcaatggctacag-3'). Crude rat nuclear protein extracts were prepared from adult rat tissues according to the method of Guerin and co-workers (36).

The complementary single-stranded oligonucleotides were synthesized on an Applied Biosystems PCRmate synthesizer for use in production of the double-stranded EMSA probes (sense strand sequences shown in Fig. 3A). Competition experiments were performed with unlabeled specific or unrelated size-matched DNA fragments. Antibody recognition experiments were performed with a polyclonal antibody to the EF domain of RXRs (kindly provided by Dr. Ellen Li, Washington University, St. Louis, MO), the D domain of PPAR\(\alpha\) (kindly provided by Dr. Michael Arand, University of Mainz, Mainz, Germany; Ref. 37), hepatocyte nuclear factor-\(\delta\) (kindly provided by Dr. Frances Sladek, University of California, Riverside, CA; Ref. 38), human COUP-TF (kindly provided by Dr. Bart O'Malley, Baylor College of Medicine; Ref. 39), and a polyclonal antibody raised to an epitope corresponding to amino acids 144-162 of TRs and a polyclonal antibody raised to an epitope corresponding to amino acids 62-82 of TR\(\beta\) (Affinity Bioreagents).

*Plasmid Constructs and Expression Systems—* Human RXRs and human RAR\(\alpha\) and human RAR\(\beta\) were overexpressed in bacterial expression vectors (pT7lac-RXR\(\alpha\) and pT7lac-RAR\(\beta\), respectively) kindly provided by David D. Moore, Baylor College of Medicine. The expression system has been described (22). Recombinant RXRs, RXRa, RXR\(\beta\), and RXR\(\gamma\) were produced by in vitro transcription/translation in rabbit reticulocyte lysates (Promega) according to the manufacturer's protocol using plasmids pSG5RXR\(\alpha\), \(\beta\), and \(\gamma\) generously provided by Dr. Pierre Chamoun (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France). The RXR\(\alpha\)-AB protein (produced in a bacterial expression system) was kindly provided by Dr. Ellen Li. RXR\(\alpha\)-AB is a GST fusion protein lacking amino acids 1-135 of the human RXR\(\alpha\) protein.

*Ultraviolet Light Cross-linking Experiments—* To isolate DNA-protein complexes, EMSA were performed as described above but scaled up 3-fold. UV cross-linking experiments were performed as described (40) with the following modifications. Following electrophoresis, the wet gel was removed from one plate, wrapped in Saran wrap, and exposed to X-Omat AR film (Kodak) at 4 °C for 1 h to determine the locations of the desired complexes that were excised and subjected to UV irradiation (6000 \(\mu\)J/cm\(^2\) × 100; Stratalinker, Stratagene). The gel slices were crushed and incubated at room temperature for 1 h in an elution buffer consisting of 0.1% SDS, 50 mM Tris, pH 7.9, 0.1 mg/ml bovine serum albumin 0.2 M NaCl, 50 \(\mu\)l of 2× Laemmli sample buffer (0.2 M Tris-HCl, pH 6.8, 3% SDS, 30% glycerol, 0.005% bromphenol blue, 3% \(\beta\)-mercaptoethanol) was added, and the samples were boiled for 3 min. The supernatants were run on a 5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane for autoradiography.

*Protein Immunoblot Analysis—* A modification of the protein immunoblot (Western) analysis described by Burnette (41) was performed using the Enhanced Chemiluminescence detection system (Amersham Pharmacia Biotech). Specific protein antigens were identified by use of a polyclonal antibody to the EP domain of RXRs kindly provided by Dr. Ellen Li.

*Experimental Animals—* The harvest of tissues from adult rats was conducted in strict accordance with the National Institutes of Health guidelines regarding humane treatment for the care and use of laboratory animals. The use of animals for isolation of nuclear protein extracts was approved by the Animal Care Committee of the Institutional Review Board of Washington University.

**RESULTS**

An RXR-dependent, Cardiac-specific Complex Forms on the Retinoid Response Element, NRRE-1—DNA-protein binding studies were performed to identify single-stranded oligonucleotides that interact with the RXRs on the pleiotropic retinoid-responsive element, NRRE-1. EMSA were performed with a double-stranded NRRE-1 probe and crude cardiac nuclear protein extracts in the absence or presence of bacterially overexpressed RXRs. In the absence of exogenously added RXRs, long exposure (24–48 h) of the EMSA autoradiograph demonstrated that two NRRE-1-protein complexes formed with the cardiac nuclear protein extract (data not shown). Previous antibody recognition studies have shown that the two faint NRRE-1-cardiac nuclear protein complexes contain homodimers of the orphan nuclear receptors, COUP-TF and estrogen-related receptor \(\alpha\) (26, 33), but not...
RXR. However, addition of increasing amounts of RXRα to the sample containing the cardiac nuclear protein extract and the NRRE-1 probe resulted in the formation of a prominent RXR-dependent complex of low mobility (Fig. 1, lane 4). The low mobility RXR-dependent complex did not form on NRRE-1 in the absence of cardiac nuclear proteins (Fig. 1, lane 3). Competition experiments with a molar excess of unlabeled double-stranded NRRE-1 DNA or a size-matched, unrelated DNA fragment (Fig. 1, lanes 5–8) confirmed that the RXR-dependent, cardiac nuclear protein-dependent NRRE-1-protein complex was specific. Addition of the RXR ligand, 9-cis retinoic acid, did not significantly alter the formation of the NRRE-1-protein complex (data not shown).

To determine whether the formation of the low mobility RXR-dependent NRRE-1-protein complex was unique to cardiac-derived nuclear proteins, the EMSA were performed with nuclear protein extracts prepared from adult rat brain or liver. Although two light NRRE-1-protein complexes were observed with protein extracts derived from all three tissues in the absence of exogenously added RXRα, in the presence of added RXRα the prominent low mobility complex was only observed with extracts derived from heart (Fig. 2, lanes 1 and 2 compared with lanes 3–6). Moreover, the RXR-dependent complex did not form with nuclear protein extracts prepared from rat skeletal muscle, NIH 3T3 fibroblasts, or the HIB brown adipocyte line (data not shown). These data identify a cardiac-enriched RXR interacting protein.

**Fig. 1.** An RXR-dependent cardiac protein-DNA complex forms on the retinoid response element NRRE-1. An autoradiograph of EMSA performed with a 32P-labeled NRRE-1 oligonucleotide probe is shown. The probe was incubated with 20 μg of crude rat cardiac nuclear protein extract (CNE) and recombinant RXRα (200 ng of extract produced in bacteria) as indicated at the top. Competition (COMP) experiments were performed with 500, 200, and 100 molar excess of unlabeled NRRE-1 (SP) or 500 molar excess of a size-matched, unrelated double-stranded oligonucleotide (NS) as indicated.

**Fig. 2.** Formation of RXR-CERIP-NRRE-1 requires cardiac nuclear proteins. An autoradiograph of EMSA performed with NRRE-1 probe and 20 μg of crude nuclear protein extract (EXT) prepared from adult rat heart (C), brain (B), or liver (L) with (+) or without (−) addition of 200 ng of RXRα-containing extracts.

**Fig. 3.** RXR-CERIP binds to an everted imperfect repeat comprised of hexameric binding sites 1 and 2 within NRRE-1. A, sense strand DNA sequences of normal (top) and mutant MCAD NRRE-1 oligonucleotides used to produce the probes used in the EMSA shown in Fig. 3B. Arrows and corresponding numbers represent location and relative orientation of potential hexameric nuclear receptor-binding half-sites (23). The single base pair substitutions in the mutant probes M1, M2, and M3 are underlined. The lowercase letters denote overhangs used for “fill-in” labeling. B, a representative autoradiograph of EMSA performed with crude cardiac nuclear protein extract (20 μg), RXRα produced in bacteria (200 ng of extract), and labeled NRRE-1 (WT) or mutant NRRE-1 probes (Δ1; Δ2,3; M1; M2; and M3). RC denotes the RXR-CERIP-NRRE-1 complex.

The Interaction of RXR-CERIP with NRRE-1 Requires a Nuclear Receptor Recognition Site—We have shown previously that NRRE-1 is a complex nuclear receptor response element that contains three potential nuclear receptor-binding sites (Fig. 3A, sites 1, 2, and 3). EMSAs were performed with a series of mutant NRRE-1 probes to delineate the binding site requirements for the RXR-CERIP-DNA complex (complex RC). Complex RC did not form with mutant NRRE-1 probes containing a deletion of site 1 (Δ1) or a deletion of sites 2 and 3 (Δ2, 3, Fig. 3B, lanes 2 and 3). To explore further the requirements for sites...
were performed with the NRRE-1 probe incubated with RAR
and relative amount of the
in vitro
lanes 1–3

To determine whether
EMSA were repeated with
the formation of complex RC is dependent on RXR subtype, the
Complex RC did not form with M1 or M2 but did form with the
binding site mutation abolishes nuclear receptor binding (23).
binding sites (Fig. 3
formed with point mutant NRRE-1 probes (M1, M2, and M3),
receptor-binding half-sites matching the consensus, RGGTTNA.
Vertica
lines
denote sequence identity, and the
underlined nucleotide
indicates the position mutated in the M1 probe. The autoradiograph depicts
the result of EMSA performed with hNRRE-1 (positive control) or
mNRRE-1 probes using cardiac nuclear extract (CNE) and bacterially
produced RXRα as described above.

1–3 in the RXR-CERIP-NRRE-1 interaction, EMSA were performed
with point mutant NRRE-1 probes (M1, M2, and M3),
each containing a cytidine substitution for the “invariant” second
position guanine within one of the three potential hexamer
binding sites (Fig. 3A). We have shown previously that each
binding site mutation abolishes nuclear receptor binding (23).
Complex RC did not form with M1 or M2 but did form with the
M3 probe (Fig. 3B, lanes 4–6). Thus, NRRE-1 site 3 is dispensable,
but sites 1 and 2, which comprise an imperfect everted
repeat separated by 8 base pairs, are required for the formation
of complex RC.

To determine whether the RXR-CERIP complex recognized
other RXR response elements, EMSA were repeated with a
probe containing a known RXRE comprised of a direct repeat
sequence separated by a single base pair (DR-1; 5'-gatctaggt-
caaaggtcatctag-3'). A second probe containing NRRE-3, a
nuclear receptor response element distinct from NRRE-1 present in
the human MCAD gene (sense strand sequence, 5'-gatecgag-
ttagtcaaggccgtgacccgtgtg-3'; Ref. 42) was also used in these
experiments. Neither the DR-1 nor the NRRE-3 probe formed
the RXR-CERIP complex (data not shown). In contrast, a probe
containing a homologue of NRRE-1, previously identified in the
mouse MCAD gene promoter region (mNRRE-1; Ref. 35)
formed an RXR-dependent, cardiac nuclear protein-dependent
complex of identical mobility to that of complex RC (Fig. 4). The
mNRRE-1 DNA sequence contains significant homology with
that of the human NRRE-1 (Fig. 4) but contains only one class
II nuclear receptor-binding half-site consensus sequence (RG-
GTTNA). Taken together, these results indicate that
RXR-CERIP binds specific recognition sites within the mouse
and human MCAD gene promoter regions, each containing an
RXR-binding half-site and additional upstream sequence
requirements.

The RXR-CERIP-NRRE-1 Interaction Is RXR Subtype-specific and Requires the RXR AB Region—To determine whether
the formation of complex RC is dependent on RXR subtype, the
EMSA were repeated with in vitro translated murine RXRa, β,
or γ in the presence of cardiac nuclear protein extract (Fig. 5A,
lanes 1–3). Complex RC formed with RXRa but not RXRβ and
was barely detectable with RXRγ. To control for the integrity
and relative amount of the in vitro translated proteins, EMSA
were performed with the NRRE-1 probe incubated with RARβ
(produced in bacteria) in the presence of equivalent amounts of
each of the RXR subtypes. We have shown previously that
RXR-RAR heterodimers bind to NRRE-1 (22). The intensity of the

FIG. 4. The RXR-CERIP forms on the mouse NRRE-1. The mouse
(mNRRE-1) and human (hNRRE-1) NRRE-1 sense strand sequences
are shown at the top. The arrows denote potential class II nuclear
receptor-binding half-sites matching the consensus, RGGTTNA. Vertical
lines denote sequence identity, and the underlined nucleotide
indicates the position mutated in the M1 probe. The autoradiograph depicts
the result of EMSA performed with hNRRE-1 (positive control) or
mNRRE-1 probes using cardiac nuclear extract (CNE) and bacterially
produced RXRα as described above.

FIG. 5. The RXR-CERIP-NRRE-1 interaction is RXR subtype-specific and requires the RXR AB region. A, autoradiograph of
EMSA performed with cardiac nuclear protein extract (CNE; 20 μg), RXR α, β, or γ produced by coupled in vitro transcription/translation,
and RARβ (produced in bacteria) as denoted at the top. Equivalent
amounts of lysate containing RXRa (lanes 1 and 4), RXRβ (lanes 2 and
5), or RXRγ (lanes 3 and 6) were added. The lower panel depicts a long
exposure of the same autoradiograph. B, autoradiograph of an EMSA
performed with the NRRE-1 probe and various combinations of RARα
(RAR), RXRα, cardiac nuclear protein extract (CNE), and a mutant
GST-RXRα fusion protein lacking the AB domain (RXR (∆AB)) as
indicated at the top. The ramp symbols over lanes 5 and 6 and lanes 7
and 8 denote increasing protein to DNA ratio varied by changing the
amount of poly(dI-dC) added while keeping nuclear receptor protein
constant.
DNA-protein complexes formed with RXR-RAR were similar among each of the RXR subtypes (Fig. 5A, lanes 4–6), confirming that equivalent amounts of receptor were present in each lane. The results shown above indicate that the RXR-CERIP interaction is subtype-specific. The RXR subtypes exhibit high amino acid homology in the DNA-binding and dimerization/ligand-binding domains (C-F domains) (13). However, the NH$_2$-terminal AB region is more highly conserved for a given subtype between species than across subtypes within the same species (43). We therefore sought to determine whether the subtype specificity of the RXR-CERIP interaction was conferred by the RXR AB region by performing EMSA with the NRRE-1 probe and cardiac nuclear extracts in the presence of a mutant GST-RXR fusion protein in which the AB region was deleted (RXR(ΔAB)). As expected, RXR(ΔAB), which contains the DNA-binding and classic receptor dimerization domains, formed a heterodimer with RARβ to bind the NRRE-1 probe (Fig. 5B, lanes 1–4). The mobility of the RXRΔAB-RARβ-NRRE-1 complex was slightly lower than that of RXRo-RAR-NRRE-1 because of the additional mass contributed by the GST polypeptide. In contrast, the RXR(ΔAB) protein did not form complex RC over a range of protein to DNA ratios (Fig. 5B, lanes 5–8). These data confirm a requirement for the AB region of RXR in the RXR-CERIP interaction and are consistent with the observation that the formation of complex RC occurs in a subtype-specific manner.

CERIP Is a DNA-binding Protein of Approximately 110 kDa—To further characterize CERIP, antibody recognition EMSA were performed with antisera raised to a variety of RXR partners and homodimeric orphan receptors shown previously to bind NRRE-1 in vitro. The DNA-protein complex supershifted with the addition of antiserum to the EF domain of RXRα (Fig. 6A, arrow, lane 7) but not with the addition of antisera raised to PPARα, TRβ, RARβ, and COUP-TF (Fig. 5A, lanes 3–6). Parallel experiments also demonstrated that antibodies to TRα or HNF-4 did not recognize proteins within complex RC (data not shown). These data, the AB domain dependence of the RXR-CERIP interaction, and the observation that mNRRE-1 forms complex RC despite containing only one nuclear receptor half-site binding sequence suggest that the CERIP is a potentially novel, non-nuclear receptor RXR interacting protein.

To determine whether a known RXR heterodimeric partner would compete with the RXR-CERIP complex for binding to NRRE-1, EMSAs were performed with the addition of increasing amounts of RARβ overproduced in bacteria to a mixture containing the NRRE-1 probe, cardiac nuclear protein extract, and RXRα. Addition of RARβ led to the appearance of a faster migrating complex shown previously (22) to consist of RXR-RARβ-NRRE-1 (open arrowhead, Fig. 6B). The intensity of complex RC (closed arrowhead, Fig. 6B) decreased coincident with the formation of the RXR-RARβ-NRRE-1 complex. These results indicate that RAR competes with CERIP to interact with RXR.

RXR has been shown to heterodimerize with a variety of nuclear receptor dimeric partners as well as an increasing number of co-activators and co-repressors. The data shown above do not distinguish between the possibilities that CERIP is a DNA-binding heterodimeric partner versus an RXR interacting protein that does not bind DNA. Indeed, both types of proteins could be present in complex RC. To determine whether CERIP is a DNA-binding protein, UV cross-linking studies were performed. For these studies, complex RC (Fig. 7A, lane 1) was excised from a non-denaturing EMSA gel, subjected to UV irradiation, and eluted from the gel. The eluted sample was separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was used for autoradiography and immunoblot analysis. A RAR-RXR-NRRE-1 sample was used as a positive control for the RXR band (Fig. 7A, lane 3). In addition, the same area of the gel in a lane containing NRRE-1 probe and cardiac nuclear extracts without RXRα was excised (Fig. 7A, lane 2), subjected to UV irradiation, and eluted from the gel to be used as a negative control. Autoradiography demonstrated that a 56-kDa signal was present in the positive control sample and in the sample containing complex RC (Fig. 7B, upper panel, open arrowhead, lanes 1 and 3) but not in the negative control lane (lane 2). An additional band of approximately 130 kDa was also present in the sample containing complex RC (Fig. 7B, upper panel, closed arrowhead, lane 1) but not in other lanes. This latter band represents a second NRRE-1-binding protein, the putative cardiac RXR interacting protein, CERIP. The calculated mass of CERIP after correction for the size of the double-
consistent with a proteolytic breakdown product of the RXR.
However, the anti-RXR antisera did not recognize the 110-kDa protein, excluding the possibility that it represents a doubly cross-linked RXR heterodimer or multimerized RXR (Fig. 7B, lower panel, lane 1). These results identify CERIP as a 110-kDa DNA-binding protein.

**DISCUSSION**

The identification of cardiac RXRα target genes involved in contractile function and mitochondrial energy metabolism has defined an important role for this nuclear receptor transcription factor in the postnatal mammalian heart. In this report, we describe a CERIP that has several unique features. CERIP is a DNA-binding protein that interacts with RXR in a subtype-specific manner via the amino-terminal AB region. The size of CERIP, based on UV cross-linking studies, is significantly larger than that of other known RXR dimeric partners, suggesting that RXR is capable of dimerizing with transcription factors distinct from the nuclear receptor superfamily via a novel interaction domain. These results extend our understanding of the functional role of the RXR AB region, suggest a mechanism for the cardiac and subtype specificity of retinoid signaling and identifying a potentially new level of regulatory complexity for RXR-mediated transcriptional control.

The RXR-CERIP interaction requires the RXR amino-terminal AB region and is RXR subtype-specific. This result was surprising because most RXR-protein interactions occur via the carboxyl-terminal DEF region of the molecule. The DEF region of RXR contains domains necessary for ligand binding, dimerization with nuclear receptor partners, and for interaction with a variety of recently identified co-activators and co-repressors such as N-COR (44), SMRT (10), RIP-140/RIP-160 (6), SRC-1 (45), and others. The amino acid sequence of the AB region of RXR is highly variable among RXR subtypes but exhibits striking conservation within subtypes between species (13), suggesting an important functional role for this domain. Our results indicate that the AB region contains a domain or domains that provides an interface for interaction with other DNA-binding proteins. The size of CERIP, based on our UV cross-linking studies, is significantly larger than that of other known members of the nuclear receptor superfamily, suggesting that in certain cellular contexts, RXR is capable of dimerizing with factors, other than nuclear receptors, via a novel interaction domain. This conclusion is further supported by the observation that complex RC also forms with the mNRRE-1, an element with only a single nuclear receptor-binding half-site. Several recent studies have demonstrated that IκB proteins interact with RXR via the AB domain. Cell culture co-transfection studies demonstrated that the IκB protein, Bcl3, co-activates the 9-cis-retinoic acid-induced transactivation of RXR (46), whereas a second IκB protein, IκBβ, inhibits its actions (47). In a separate study, the transcription factor myocyte-specific enhancer factor 2 was shown to interact with the thyroid receptor to regulate α-myosin heavy chain gene promoter activity (48). Collectively, these results suggest that in addition to hetero- and homodimeric interactions with nuclear receptors via the classic dimerization domain, RXR dimerizes with distinct subsets of transcription factors via domains within the AB region to mediate transcriptional control and that this may occur in a subtype- or isoform-specific manner.

The AB region of RXR has also been shown to possess a ligand-independent transcriptional activating (AF-1) function (49). Recent studies have indicated that RXR isoforms and subtypes possess cell- or tissue-specific AF-1 properties. Chamblon and co-workers (50) have recently described a new RXR isoform (mRXRα2/3) that is expressed specifically in the mouse testis and appears to have distinct AF-1-mediated transcrip-
tional regulatory properties when compared with that of mouse RXRa1. The RXRγ AB region was shown to confer transcriptional activation in a myocyte-specific manner (51). Our data suggest that domains within the RXR AB region may confer cardiac-specific transcriptional regulatory properties to RXRα and RXRγ via interaction with CERIP. It will be of interest to identify CERIP and evaluate its functional effect on RXR-mediated transcriptional control.

CERIP heterodimerizes with RXR to bind NRRE-1, a complex retinoid response element in the promoter region of the human MCAD gene (22). Cell culture co-transfection experiments and DNA-protein interaction studies have demonstrated that NRRE-1 is a pleiotropic element capable of interacting with a variety of nuclear receptors, including RXR-PPARα, CYP-TP I and II, and HNF-4 (23, 25, 34). Studies performed with mice transgenic for fragments of the human MCAD gene promoter fused to reporter genes have shown that NRRE-1 is required for appropriate expression of the MCAD gene among tissues and during prenatal cardiac development (26). These results have suggested that the complex architecture of NRRE-1 provides a mechanism for multiple upstream signaling pathways to converge on a single transcriptional regulatory element to regulate MCAD expression in vivo. The identification of a cardiac-specific, RXR-dependent NRRE-1-binding protein suggests a mechanism for the cardiac-enriched expression of the MCAD gene and identifies a potentially novel level of regulatory complexity related to RXR-mediated transcriptional control. Consistent with this possibility, we found that the RXR-CERIP-NRRE-1 complex did not form in noncardiac tissues with lower MCAD expression such as brain and liver.

Our UV cross-linking studies do not exclude the possibility that, in addition to CERIP, non-DNA-binding proteins may interact with RXR in the CERIP-RXR-NRRE-1 complex. We were surprised that our DNA-protein binding studies did not detect PPARα within the CERIP-RXR complex, given that it is known to bind NRRE-1 as an RXR partner (25) and is expressed in heart (55). It is possible that the cross-linking efficiency of PPARα to NRRE-1 is low compared with that of RXRα and CERIP or that loss of the PPAR ligand during nuclear extraction resulted in a loss of heterodimerization with RXRα and a reduced affinity for NRRE-1. In any case, our results identify a novel RXR protein interaction with NRRE-1.

In summary, our findings provide evidence for a novel cardiac-enriched retinoid X receptor partner termed CERIP. The CERIP-RXR interaction is RXR isoform-specific and RXR AB domain-dependent. We speculate that CERIP co-regulates the expression of the RXRα and RXRγ target genes in heart.

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