INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Characterization of CIA (Coactivator Independent of Activation Function-2), a Novel Nuclear Receptor Coactivator

Frédéric Sauvé

Department of Biochemistry
McGill University
Montréal, Québec
November 1999

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

ABSTRACT

Coactivators for the superfamily of nuclear receptors are defined as factors that enhance their transcriptional activity. Most of these coactivators exert their action through the receptor ligand-dependent activation function 2 (AF-2). This interaction occurs between a coactivator LXXLL motif (NR-box) and a hydrophobic cleft located on the ligand-binding domain of the receptor. Here we describe the cloning and characterization of CIA, a novel Coactivator Independent of AF-2 function. CIA displays specific interaction with the RVR orphan nuclear receptor and both specific interaction and transcriptional coactivation potential with the estrogen receptors (ER) α and β. The interaction with ERα and β is strongly enhanced by its natural ligand, estradiol (E_2) and surprisingly also by pure antiestrogens EM 800 and ICI 164,384. While the E_2-dependent CIA-ERα interaction requires an intacte CIA LXXLL motif, CIA also interacts with an AF-2 null mutant of ERα. Therefore, CIA constitutes the first example of a novel type of ligand-dependent but AF-2 independent nuclear receptor coactivator that may play a specific role in the ER physiology via selective ER modulators (SERMs).

RÉSUMÉ

Les coactivateurs pour la superfamille des récepteurs nucléaires sont définis comme étant des facteurs augmentant leur activité transcriptionelle. La majorité de ces coactivateurs exercent leur action sur la fonction d'activation 2 (AF-2) du récepteur, dont l'activité nécessite la présence du ligand. Cette interaction se produit entre une portion hydrophobe de la surface du récepteur, située dans le domaine de liaison du ligand (LBD) et un motif présent à la surface du coactivateur, dont la séquence est LXXLL. Dans cette étude, nous décrivons l'identification et la caractérisation d'un nouveau coactivateur nommé CIA (Coactivator Independent of AF-2). Cette protéine interagit fortement avec un récepteur orphelin (RVR) ainsi qu'avec les deux récepteurs des estrogènes (ERα et β). Cette interaction est grandement amplifiée par la présence du ligand et dépend de l'intégrité du motif LXXLL de CIA. De plus, cette interaction est favorisée par des antiestrogènes "purs" tels que EM800 et ICI 164,384 et est indépendante de l'intégrité de l'AF-2. Par le biais d'expériences de co-transfections, on note que la présence de CIA augmente spécifiquement l'activité transcriptionelle de ERα et β. Nous croyons donc que CIA est un nouveau type de coactivateur dont l'action est dépendante de la présence du ligand, mais indépendante de l'intégrité de l'AF-2 et qui pourait jouer un rôle spécifique dans la physiologie des estrogènes.

PREFACE - Contribution of Authors

The research presented in this thesis is entirely my own with the following exceptions:

The initial cloning of CIA was done by Dr. Linda D.B. McBroom in a yeast two-hybrid assay.

In the manuscript presented as the core of this thesis, the sequence in figure 1a, the liquid β-galactosidase assays performed in figure 2, the northern blots on figure 3a and the pull-down on figure 6a were all done by Josette Gallant and previously presented in her thesis (Gallant, 1997). The manuscript was entirely written by me with editorial comments and corrections by Dr. Vincent Giguère.

ACKNOWLEDGMENTS

I would like to acknowledge my supervisor, Dr. Vincent Giguère, for his support, his presence as a guide and for providing me with the opportunity to work in his laboratory.

I would also like to acknowledge all the members of the laboratory who provided me with support and entertainment. I am especially thankful to Dr. Robert Sladek for his discussions, criticism, and ability to provide extremely useful advice on both experimental design and execution. He was also the major contributor to the proper english revision of this thesis. Thanks also to Anna Moriatis for her support as a fellow student, her critiques and encouragement were always to the point. Special thanks also to Dr. Darren Kamikura first for his friendship and second, for his help with the proper phrasing of this thesis introduction. Without him the time spent in and out of the lab would have been less enjoyable.

I also want to acknowledge Dr. Alain Nepveu, Dr. Morag Park, Brigitte Goulet and Hanane Khoury who generously provided me with the various cancer cell lines.

Finally, I would like to give a very special thanks to Anne-Marie Pulichino, for her understanding and compassion throughout the last two years, and especially during the writing of this thesis. She also helped me maintain an adequate balance between work and life outside the laboratory. I sincerely believe that this was, more than anything else, what allowed the completion of this work.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

35S	sulfur-35
AA	amino acid
AF-1	activation function-1
AF-2	activation function-2
AIB-1	amplified in breast cancers-1
AR	androgen receptor
ARA	androgen receptor activator
BAC	bacterial artificial chromosome
bHLH	basic helix-loop-helix
bp	base pair
cAMP	cyclic adenosine-3', 5'-monophosphate
CARM1	coactivator-associated arginine methyltransferase 1
CBP	CREB-binding protein
cDNA	complementary DNA
CIA	coactivator independent of activation function 2
CREB	cAMP response element-binding
CTE	carboxy-terminal extension
DBD	DNA-binding domain
DNA	deoxyribonucleic acid
DRIP	vitamin D receptor interacting protein
E2	17β-estradiol
EGF	epidermal growth factor
ER	estrogen receptor
ERAP	estrogen receptor associated protein
ERE	estrogen response element
EST	expressed sequence tagged
FISH	fluorescence in situ hybridization
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gDNA	genomic DNA
GR	glucocorticoid receptor
GRIP	glucocorticoid receptor interacting protein
GST	glutathione s-transferase
GTF	general transcription factor
HAT	histone acetyl-transferase

HDAC	histone deacetylase complex
HNF-4	hepatocyte nuclear factor-4
HRE	hormone response element
HSP	heat shock proteins
HTGS	high-throughput genome sequencing
IGF-1	insulin growth factor-1
kb	kilo base (pairs)
KDa	kilodaltons
KRAB	kruppel-associated protein b
LBD	ligand binding domain
M	molar
MAPK	mitogen-activated protein kinase
MDa	megadaltons
MR	mineralocorticoid receptor
mRNA	messenger RNA
NCBI	national center for biotechnology information
NCoA	nuclear receptor coactivator
NCoR	nuclear receptor corepressor
NR	nuclear receptor
P/CAF	p300/CBP associated factor
p/CIP	p300/CBP interacting protein
PAS	per/arnt sim
PCR	polymerase chain reaction
PHD	plant homeodomain
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
RA	retinoic acid
RAC	receptor associated coactivator
RACE	rapid amplification of cDNA ends
RAGE	rapid amplification of genomic ends
RAR	retinoic acid receptor
RIP	receptor-interacting proteins
RNA	ribonucleic acid
RT	reverse transcriptase
RXR	retinoid x receptor
SERM	Selective estrogen receptor modulator

SF-1	steroidogenic factor-1
SMCC	SRB- and MED-containing cofactor complex
SMRT	silencing mediator for retinoid and thyroid hormone receptors
SRA	steroid receptor RNA activator
SRC-1	steroid receptor coactivator-1
STS	sequence tagged sites
TAF	TATA-binding proteins-associated factor
TBP	TATA-binding protein
TIF-1	transcription intermediary factor-1
TR	thyroid receptor
TRAM-1	thyroid hormone receptor activator molecule-1
TRIP	thyroid receptor interacting protein
UTR	untranscribed region
VDR	vitamin D receptor

CHAPTER I - INTRODUCTION

1. Nuclear receptor superfamily

Nuclear receptors are a superfamily of ligand-dependent transcription factors that regulate hormonal processes involved in development, differentiation, metabolism and reproduction (Meyer et al., 1989). They can be subdivided into three main groups based on their dimerization potential, ligand-binding and DNA-binding specificity (Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995). Group I comprises receptors for steroid hormones such as estrogens (ER) (NR3A1 and NR3A2, according to the nuclear receptor nomenclature committee, 1999), progestins (PR)(NR3C3), androgens (AR)(NR3C4), mineralocorticoids (MR)(NR3C2) and glucocorticoids (GR)(NR3C1). Group II includes receptors for non-steroidal hormones such as vitamin D (VDR)(NR1I1), thyroid hormone (TR)(NR1A1 and NR1A2), all-*trans* retinoic acid (RAR)(NR1B1, NR1B2 and NR1B3) and 9-*cis* retinoic acid (RXR)(NR2B1, NR2B2 and NR2B3). The third group comprises all the receptors for which no ligand has yet been identified and are termed "orphan receptors".

Nuclear receptors share a number of common structural characteristics and consist of five functional domains (see figure 9) (Mangelsdorf et al., 1995). The first region, named the "AB" domain, is located at the amino-terminus end of the receptor. It is a very poorly conserved region, even within a sub-family of receptors, both in terms of primary sequence and length. The AB domain contains the activation function-1 (AF-1), one of the two transactivation functions of the receptor, whose activity is ligand-independent (Evans, 1988). The "AB" region has been implicated in modulating target gene specificity and influencing the DNA-binding activity of receptors. For some receptor family members, multiple modulator domains arise from a single gene through alternative

splicing, usage of different promoters and distinct translational start sites (Giguère, 1994). Recent evidence suggests that the activity of the "AB" domain is controlled post-translationally through phosphorylation in response to extracellular growth factors (Tremblay et al., 1999). Phosphorylation has many effects including recruitment of general (McInerney et al., 1996) or specific coactivators (Shao and Lazar, 1999).

The second region is referred to as the "C" domain or the DNA-binding domain (DBD). As its name implies, this region of the receptor is responsible for contacting DNA on specific sequences named hormone response elements (HREs) (Evans, 1988; Green et al., 1988). The DBD is the most highly conserved region among nuclear receptor superfamily members and consists of a pair of Cys_2:Cys_2 zinc fingers, which make direct contact with the DNA, as well as a carboxy-terminal extension (CTE) of approximately 25 residues (Wilson et al., 1992). The "C" domain is also partially responsible for the dimerization of the receptor.

The third region or "D" domain is a hypervariable region adjacent to the DBD, which is sometimes referred to as the "hinge region". Its main function is to serve as a flexible stretch between the DBD and the LBD that allows sufficient rotation for some receptors to dimerize both on direct and inverted HREs. This region has also been implicated in binding the coregulator NCoR (Horlein et al., 1995; Zamir et al., 1996), and can influence the receptor ability to bend DNA (McBroom et al., 1995; Nardulli and Shapiro, 1992).

The fourth region or "E" domain is responsible for ligand binding and transactivation. Nuclear receptors ligand-binding domains (LBDs) are moderately well conserved in terms of their primary structure: their secondary and most of their tertiary structure are remarkably well conserved, consisting of eleven to thirteen alpha-helices forming a pocket for the ligand. The individual differences in this ligand-binding pocket dictate

specificity of the receptor through space availability as well as the nature of both direct and indirect contacts made with the ligand. In absence of ligand, the last helix of the LBD (helix 12) points toward the outside of the pocket, in a conformation often referred to as "floating outside of the LBD" that inhibits receptor-coactivator interaction. Upon ligand binding, helix 12 folds back on the surface of the LBD, making contacts with helix 3, 4 and 5. This important conformational change has major implications for the transcriptional activity of the receptor, as it positions the second transcriptional activation function (AF-2), located on helix 12 (Danielian et al., 1992). To create a new interface necessary for the recruitment of coregulatory proteins. The LBD has also been implicated in a vast number of other functions including dimerization, nuclear localization and binding to heat shock proteins (HSP).

The final region, or "F"domain, is an extension to the LBD that is present only in a few receptors. So far, no correlation has been established between the presence of such extension and any functional characteristic of the receptor.

The different groups of NR's differ in their DNA binding characteristics. Group I receptors bind to DNA on palindromic repeats (AGAACA for the GR subfamily and AGGTCA for the ERs) in a homodimeric head-to-head arrangement (reviewed in Beato et al., 1995). These receptors absolutely require ligand-binding in order to bind to DNA *in vivo*. Conversely, group II receptors can bind DNA constitutively as homodimers, to direct repeats (containing response elements AGGTCA), even in absence of ligand (Beato et al., 1995; Mangelsdorf et al., 1995). They can also bind as heterodimers with the receptor for 9-cis retinoic acid (RXR), either to direct or everted repeats (Glass, 1994). This heterodimerization potential with RXR allows an additional level of control through the possible modulation of the amplitude of transcriptional response with the ligands of both RXR and its partner. The type of dimerization depends on the nature of the receptor

and on the structure of the HRE it contacts (Schwabe et al., 1993). Finally, some receptors can bind DNA as monomers on a half-site that is preceded by a 5'-AT rich sequence (Giguère et al., 1995; Giguère et al., 1994; Harding and Lazar, 1993; Wilson et al., 1993). The binding specificity for each family of both homo- and heterodimeric receptors depends on the spacing between the half-site repeats on the DNA.

2. Orphan nuclear receptors

Orphan receptors constitute a subfamily of the nuclear receptor superfamily that comprises receptors for which no ligand as yet been identified. Most of these receptors have been identified through their structural homology with known hormone receptors (Giguère, 1999). The number of orphan receptors is constantly on the rise and novel ones are identified on a regular basis. As of recent count, the number of orphans is easily double the number of receptors with a known ligand (Willy and Mangelsdorf, 1998). A common way to discover a new orphan receptor is to perform low homology screening of a cDNA library, using the well conserved DBD as a probe. Another method is to perform PCR techniques using degenerate primers (Enmark and Gustafsson, 1996). More recently, new *in silico* approaches have been used to identify novel gene products that are homologous to known nuclear receptors. For example, the pregnane receptor PXR (NR1I2) has recently been identified via motif searches of public EST databases (Kliewer et al., 1998).

After the identification of a novel orphan receptor, three major goals are to establish the functional properties of this protein, evaluate whether it is hormone responsive or not and define its physiological role *in vivo* (reviewed in Sladek and Giguère, 1999). The functional properties are usually easily established through studies of the receptors

predicted peptide sequence, as well as by DNA binding and dimerization studies (Sladek and Giguère, 1999). With the knowledge of their DNA binding properties and their requirement of heterodimer partners (such as RXR, the receptor for 9-cis retinoic acid), most of the orphans can be placed into one subgroup of the superfamily (Leblanc and Stunnenberg, 1995).

Researchers identifying a novel orphan receptor are facing a singular but often arduous task: identification of a ligand. Establishing whether an orphan receptor is ligand-responsive is a controversial question (O'Malley and Conneley, 1992). In the field of orphan receptors, it has been suggested that the majority of receptors indeed have ligands awaiting discovery (Blumberg and Evans, 1998). A common method used to identify novel ligands is the cotransfection screening assay (Kliewer et al., 1999). This is a cell culture approach in which the orphan receptor activates transcription of a reporter gene in the presence of a putative ligand (Giguère et al., 1986). Some pharmaceutical companies use this technique to screen chemical compound databases. The problem associated with these massive screens is that they most often lead to synthetic compounds that can be considerably different from the natural ligand. Despite that, these screens are generally useful because they can lead to ideas of what the natural ligands may be. In order to identify natural ligands for a given receptor, the researcher often needs to focus on specific pathways, based on data obtained via study of the physiology of the receptor.

Defining the physiological role of a novel orphan receptor is also a complex question. Some insights may be acquired via study of expression patterns. Sometimes the presence of the mRNA in specific tissues is a potent indicator of its role. Another useful tool is genetic ablation of the studied orphan receptor in cell lines and mice (Kastner et al., 1995). These resources allow the identification of phenotypic differences presumably caused by the absence of the receptor. They also give a good basis for the identification of

putative target genes for the orphan receptor. The possibilities are vast and researchers usually combine information obtained from all available sources in order to draw a picture of the receptor's physiological role. Recently, novel approaches have been developeded to identify putative ligand for a known orphan receptor. This concept of first identifying a receptor and then to search for its cognate ligand is termed reverse endocrinology (Giguère, 1999; Kliewer et al., 1999).

3. The estrogen receptors

The identification and characterization of a cellular receptor for the female sex hormone 17β-estradiol (E_2) dates back to the 1960's (Toft and Gorski, 1966). Molecular and biochemical studies of the estrogen receptor (ER) have been facilitated since the cloning of the cDNA for ER (Greene et al., 1986; Walter et al., 1985). Given the importance of E_2 in the development of reproductive tract and mammary gland as well as its roles in neuroendocrine regulation, cardiovascular disease and behavior (Couse and Korach, 1999), the ER attracts a lot of attention. Recently, a second receptor that responds to E_2 has been identified (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). This discovery forced the renaming of the original receptor as ERα and the new one as ERβ. More importantly, it stimulated the estradiol field and forced researchers to re sketch their model of estradiol action to include this new variable (Giguère et al., 1998).

More and more evidences indicate different and perhaps complementary roles for both ERs. Recently, analysis of functional target genes was established in an effort to compare their respective influence on transcription regulation (Vanacker et al., 1999). Somewhat surprising, the results indicated that ERα has probably evolved to regulate a broader set of target genes (being able to regulate both EREs and SF-1 response elements) whereas

ERβ's influence appeared more restricted (only through EREs). Another major difference was found in the identification of ERβ as the more potent candidate for estrogen-targeted therapy of cardiovascular diseases (Makela et al., 1999). More and more of these type of comparative studies will be required before a definite picture of their relative roles can be established.

Multiple studies have linked breast and ovarian cancer with ER status (Davidson, 1998; Ferguson et al., 1998; Leygue et al., 1998; Murphy et al., 1998), suggestion that the control of ER expression and activation is of primary importance in understanding these tumors. Apart from ligand induction, many other control mechanisms for ER activation have been discovered. Recruitment of coactivator proteins to the receptor AF-2 are known to potentiate its ligand-dependent transcription (Feng et al., 1998; Shibata et al., 1997). Therefore, amplification and/or overexpression of such coactivators can contribute to malignancies (Anzick et al., 1997; Bautista et al., 1998; Ghadimi et al., 1999). Another ER control mechanism is the phosphorylation of its AF-1 which has been linked to ligand-independent activation of the receptor downstream of growth factors (Aronica and Katzenellenbogen, 1993; Bunone et al., 1996; Kato et al., 1995). Recently, ligand-independent activation has been linked to the recruitment of coactivators, such as SRC-1, to the AF-1of the ERs (Tremblay et al., 1999). Cancer cells often demonstrate aberrant EGF or IGF signaling (reviewed in Ellis et al., 1998): inappropriate activation of ERs may be an important pathway transducing this event.

The current consensus mechanisms for ERα activation are summarized in figure 10 along with the different blocks caused by antiestrogens. The AF-1 is mainly activated by phosphorylation by the MAPK pathway, downstream of growth factors, while AF-2 activation primary depends on estradiol binding and subsequent coactivator recruitment: both these different processes can be blocked by antiestrogens. The so called "pure

antagonists" EM800 and ICI 164,384 are able to prevent activation of both AF-1 and AF-2 and also prevent coactivator binding (SRC) (MacGregor and Jordan, 1998; Tremblay et al., 1998). The partial antagonist 2-hydroxytamoxifen (OHT) causes an incomplete block of estradiol action on the AF-2 but prevents SRC binding (Grese et al., 1997). The OHT action is less efficient because it is promoter and cell-type dependent (Paech et al., 1997). Although OHT is widely used clinically in the treatment of breast cancers it has been shown to increase incidence of endometrial carcinomas via its agonist effects (Fornander et al., 1989).

4. Coactivators

Initially, nuclear receptor action could be summarized by the following model: the receptor's ligand, which is a small lipophilic molecule, would diffuse freely across the cell membrane and reach its cognate receptor in the cell. Unliganded receptor would be either in the nucleus (some even pre-bound to DNA) or complexed with heat-shock proteins (HSP) in the cytoplasm. In the latter case, ligand-binding would induce the release of the HSP complex, dimerization of the receptor and its translocation into the nucleus. Once in the nucleus, the liganded receptor would bind with high affinity to specific sites in chromatin and regulate transcription of downstream target genes (Evans, 1988).

While this simple model is still a valid guide to what is happening in the activation of nuclear receptors, but recent progress has identified sevral multiprotein complexes that play important roles in modulationg NR activity (figure 11). In absence of ligand a complex of corepressors (NCoR, SMRT, SIN3 and HDAC) and a complex of coactivators (CBP, P/CAF, P/CIP and SRC-1) both interact with the receptor, resulting in

a minimal level of transcription. This low transcription is often referred to as "basal transcription". ER can be activated either ligand dependently or independently. In absence of ligand, activation comes from phosphorylation of its AF-1 and subsequent recruitment of coactivators. The more classical estradiol-induced activation favors recruitment of coactivator complexes to AF-2 and also to AF-1. Every transcription process involves both general and specific factors.

a. General transcription factors

The general transcription factors (GTF) are key to the process of transcription initiation. The transcription itself is mediated by the RNA polymerase II (Pol II) complex. (Roeder, 1996). The initial step of this activation is the binding of the transcription factor II D (TFIID) complex to the promoter, a short distance away from transcription start site. TFIID is composed of the TATA-binding protein (TBP) and a list of more than 10 TBP-associated factors (TAF$_{II}$s) (Horwitz et al., 1996; Zawel and Reinberg, 1995). Human TFIID consists of a core group of TAF$_{II}$s (TAF$_{II}$28, TAF$_{II}$100, TAF$_{II}$135 and TAF$_{II}$250) which are present in all TFIID complexes, and a promoter-specific group of TAF$_{II}$s (such as TAF$_{II}$18, TAF$_{II}$20 and TAF$_{II}$30) (Jacq et al., 1994; Mengus et al., 1995). The formation of the TFIID/TBP unit depends on the presence of TFIIA which disrupts the inactiveTFIID/TFIID homodimers to form activeTFIID/TBP heterodimers (Coleman et al., 1999). Subsequently, TFIIB is recruited to the complex and binds DNA adjacent to the TATA box (Lee and Hahn, 1995). TFIIB then recruit TFIIFα and induces the binding of RNA Pol II (Ha et al., 1993). Evidences suggest that a stable pre-formed basal-transcription complex may exist, containing a large number of proteins, including RNA Pol II and others GTFs in a pre-assembled form (Koleske and Young, 1994). This discovery of the so called "RNA Pol II holoenzyme" contrasts with this step-by-step assembly model for the transcription initiation complex. It has been proposed that

transcription of the hormone-regulated promoters by nuclear receptors may occur via control of the rate of assembly of these transcription complexes (Horwitz et al., 1996; Klein and Struhl, 1994; McKenna et al., 1999). The initiation is also dependent on the presence of TFII-I, a multifunctional protein that appears to have functions in both transcription and signal transduction via its repeat motifs (Roy et al., 1997). Recent findings suggest that TFII-I direct phosphorylation by extracellular signal regulated kinases (ERKs) plays a key role in the activation of the c-fos promoter (Kim and Cochran, 2000).

To reinforce that hypothesis, evidence of direct contact between NRs and some of the basal factors of the pre-initiation complex are numerous (reviewed in Beato and Sánchez-Pacheco, 1996). For example, the LBD of RXR interact ligand-dependently with TBP (Schulman et al., 1995) and both AF-1 and AF-2 of ERα can also contact TBP *in vitro* (Horwitz et al., 1996). Other NRs also make contact with general transcription factors: COUP-TF (Ing et al., 1992), VDR (Blanco et al., 1995), RAR (Berkenstam et al., 1992) and TRα (Hadzic et al., 1995) interact with TFIIB, AR interacts with TFIIF (McEwan and Gustafsson, 1997) and RAR interacts with TFIIH (Rochette-Egly et al., 1997). Some NRs even interact with specific TAFs: RAR, TR and VDR interact with TAF$_{II}$135 (Mengus et al., 1997), RXR, ERα and VDR interact with TAF$_{II}$28 (May et al., 1996) and VDR and TRα interact with TAF$_{II}$55 (Lavigne et al., 1999). These interactions could define a role for NR in modulating a DNA-bound ternary complex of TBP/TAF$_{II}$s, TFIIB and receptors, as was proposed at the beginning of the decade (Ing et al., 1992).

For a long time, indirect evidence pointed to the existence of proteins other then GTFs that would interact with activated receptors. The most convincing of this evidence was based on the squelching phenomenon (Meyer et al., 1989). When two NRs are cotransfected, the activation of one of the receptors diminished the activation capacity of

the second. This was indirect evidence of titration of a cellular pool of factors, necessary for the activation of both receptors. Such experiments established a functional link between the availability of coregulatory factors and the ability of the receptor to activate transcription. It was also known that interactions between NR and the basal transcription factors are insufficient to exert hormone-dependent transcriptional control (Horwitz et al., 1996). The recent identification of a new class of receptor coactivators that interact with NR and enhance their transcription, so-called coactivators, has begun answering some of the questions regarding fine transcriptional control exerted by NRs.

b. Coactivators and their mode of action

The coactivators can enhance NR-based transcription via multiple mechanisms. Their main mode of action seems to involve chromatin remodeling. An essential step of NR-mediated gene transcription is chromatin loosening through disruption of nucleosomal structure (reviewed in Jenster et al., 1997). In this context, the coactivators are acting on histones to modify their acetylation status. Many coactivators, such as SRCs, CBP/p300 and P/CAF possess intrinsic histone acetyl-transferase (HAT) activity. Others, like TIF-1α, interact directly with chromatin remodeling complexes and are likely to being them close to the NRs (Le Douarin et al., 1996). Recently, CARM1, a coactivator that methylates the histone H3 has been identified, uncovering a role for histone methylation in transcriptional activation (Chen et al., 1999).

A diversity of other control mechanism exists. Some, like the SRC family members, possess intrinsic activation domains that can be transferred to heterologous proteins and conserve their potential to coactivate transcription (Oñate et al., 1998). Although, most coactivators do not appear to possess such domains. Others, like SRA, appear to have roles more as "linkers" and stabilize interactions between NRs and other coactivators

(Lanz et al., 1999). Finally, SMCC as well as members of the DRIP and TRIP families, form part of protein complexes that act through yet unclear mechanisms. The current model stipulates that they would promote transcription via substitution for SRCs / CBP complex as a link to general initiation factors during multiple rounds of transcription (McKenna et al., 1999; Rachez et al., 1998; Yuan et al., 1998).

A constantly growing network of these coactivators exist (see table 1), most of these are poorly characterized, and not much is known about their mode of action. In the remainder of the introduction, the best characterized coactivator groups will be discussed in more detail.

c. ERAP-140 and ERAP-160

The first coactivators described for the nuclear receptors were the ER-associated proteins (ERAPs). Two proteins (ERAP-140 and ERAP-160) were purified from a ^{35}S-methionine labeled MCF-7 cell extract, through their ability to interact with the liganded-LBD of ERα (Halachmi et al., 1994). This interaction with ERα was stabilized by estrogens and destabilized by antiestrogens such as the pure antagonist ICI 182,780. Their role in regulation of ER function was implied by their ligand-dependent interaction with the receptor and by the failure of transcriptionally defective mutants of ERα to recruit them. ERAP-160 was subsequently shown to be highly similar to hSRC-1 and is now considered to be one of its splice variants (Kamei et al., 1996).

d. RIP family

The receptor-interacting proteins (RIPs) 80, 140 and 160, were originally identified as proteins interacting with transcriptionally active ERα in a far-Western experiment

(Cavaillès et al., 1994). The subsequent cloning and analysis of RIP140 demonstrated that its interaction with ERα was dependent on the integrity of ERα transcriptional activity (Cavaillès et al., 1996). Initially, RIP140 was classified as a coactivator despite marginal enhancement of ERα transactivation in a transient transfection experiment. Recent evidences derived from mouse RIP140 repression effect on TR2 orphan receptor and on RARα mediated RA-induction of a reporter gene suggested that RIP140 acts indeed as a corepressor for some members of the nuclear receptors superfamilly (Lee et al., 1998). The biochemical mechanism of mRIP140 repressive action is yet to be clearly defined but its capacity to interact with TR2 and to alter its nuclear distribution patterns might hint to a role in managing local nuclear receptors availability.

e. SRC family

Considered as the first common transcriptional mediator for nuclear receptors, hSRC-1 was cloned from a B-lymphocyte cDNA library using the yeast-two hybrid approach,with PR LBD as a bait (Oñate et al., 1995). One of the most interesting initial observations about hSRC-1 was that it could relieve the squelching of PR that was observed when both PR and activated ER were cotransfected into cell lines (Oñate et al., 1995). This demonstrated that hSRC-1 was a common coactivator recruited by both PR and ER LBD and that it was necessary for their efficient transactivation. hSRC-1 augments the transcriptional activity of PR, GR, ER, TR, RXR (Oñate et al., 1995), hepatocyte nuclear factor 4 (HNF-4) (Wang et al., 1998) and PPARγ (Zhu et al., 1996) in a hormone-dependent manner. It was also shown to be involved in ligand-independent activation of ERα and β (McInerney et al., 1996; Tremblay et al., 1999). Evidence shows that hSRC-1 is required for full synergy between AF-1 and AF-2 of AR (Ikonen et al., 1997), ER (McInerney et al., 1996) and PR (Oñate et al., 1998).

The family of hSRC-1 related proteins contains many members and used to be referred as the p160 family of coactivators, due to their approximate apparent molecular weight of 160 kDa. All the members are close homologues and are mainly alternately spliced forms or orthologous proteins identified from different mammals. In order to maintain clarity, they are now regrouped in three classes: SRC-1, SRC-2 and SRC-3, based on the classification established by Li *and al.* (Li and Chen, 1998). SRC-1 group contains hSRC-1 (Oñate et al., 1995), mSRC-1 (Zhu et al., 1996) and NCoA-1 (Torchia et al., 1997). SRC-2 regroups GRIP-1 (Hong et al., 1996), NCoA-2 (Torchia et al., 1997) and TIF2 (Voegel et al., 1996). Finally, SRC-3 comprises mouse p/CIP (Torchia et al., 1997), AIB1 (Anzick et al., 1997), RAC3 (Li et al., 1997), ACTR (Chen et al., 1997) and TRAM-1 (Takeshita et al., 1997).

SRC proteins share common motifs. They all possess glutamine-rich regions (some with poly-glutamine sequences), tandem basic helix-loop-helix (bHLH) domains as well as Per/Arnt Sim homology (PAS) domains. These domains are known to mediate homodimeric and heterodimeric interactions between proteins (Hankinson, 1995). The presence and conservation of these domains has suggested a possible cross-talk between nuclear receptors and other PAS-containing factors (Kamei et al., 1996) but this has yet to be observed. The SRCs also contain nuclear receptor interaction (NR) boxes. These boxes (also referred to as RID-Receptor Interacting Domains) are LXXLL[a] consensus motifs which have been shown to be necessary and sufficient for ligand-dependent interaction between coactivators and the AF-2 of NRs (Voegel et al., 1996; Darimont et al., 1998; Heery et al., 1997; McInerney et al., 1998; Nolte et al., 1998; Westin et al., 1998). Recent crystal-structure analyses have elucidated the mode of action of theses LXXLL motifs (Darimont et al., 1998; McInerney et al., 1998). They form a small alpha-helix that binds to the hydrophobic cleft (Feng et al., 1998), a region formed of helix 3,4,5 and 12 on the

[a] Where L is leucine and X is any amino acid

surface of the liganded LBD of NRs. These studies provide a structural explanation for the inability of the SRC family of coactivators to bind antagonist-bound nuclear receptors. Agonist binding induces the disruption of contacts between helix 12 and the hydrophobic cleft, preventing the binding of an NR-box (Nolte et al., 1998; Shiau et al., 1998) . Different coactivators possess different numbers of these NR boxes. For example, the SRC family members possess three conserved motifs except for hSRC-1 which contains a four NR-boxes. It has been proposed that the sequence surrounding the actual motif, as well as their spacing, is what confers binding specificity of a given coactivator for a subset of nuclear receptors (Darimont et al., 1998).

f. DRIPs and TRAPs

Some recent biochemical purification techniques have identified a wide range of proteins that interact with liganded nuclear receptors. Two groups essentially performed similar experiments using epitope-tagged NR to identify interacting proteins from cell extracts in presence of ligand. Freedman and colleagues used liganded VDR to recruit a complex containing more then ten proteins that were called VDR-interacting proteins (DRIPs) (Rachez et al., 1998). Roeder and colleagues purified a similar protein complex using liganded TR as a bait and called them TR-associated proteins (TRAPs) (Fondell et al., 1996; Zhang and Fondell, 1999). Both of these complexes contain essentially the same 10 proteins -ranging in size from 70 to 230 kDa- that can enhance TR and VDR transcription on a consensus response element. Strikingly, those complexes do not contain SRC family members nor do they contain CBP. They are evidences supporting the existence of more than one complex able to enhance NR transcription (McKenna et al., 1999).

g. TIF-1 family

Screening a mouse library with RAR-LBD as a bait, Chambon and colleagues have isolated a novel protein which they called transcription intermediary factor-1 (TIF-1) (Le Douarin et al., 1995). TIF-1α complements RXRγ AF-2 activity on RAR in presence of 9-*cis* RA. Additional functional interactions were also noted with ER, PR and VDR in yeast (Thenot et al., 1997). The TIF-1 family now comprises three members: TIF-1α, β (Moosmann et al., 1996) and γ (Venturini et al., 1999). They all possess a RING finger domain, a plant homeodomain (PHD) and a B-box finger, which are all domains known to mediate DNA-protein and protein-protein interactions (Freemont, 1993). They also possess a bromodomain which is typically found in SWI/SNF multiprotein complexes (Kwon et al., 1994). This complex has been shown to promote the binding of transcription factors to chromatin structures (Muchardt and Yaniv, 1993; Yoshinaga et al., 1992). TIF-1α can also interact with mHP1α and MOD1, which are heterochromatin-associated proteins (Le Douarin et al., 1995). Despite this possible association with chromatin remodeling proteins, there is a major controversy in the classification of TIF-1s as coactivators since they also display properties that would be unexpected from a coactivator: for example, TIF-1α represses transcription when fused to a heterologous DBD (Le Douarin et al., 1998). Also, in some experimental settings, TIF-1α can repress transcription by ER and RAR (Le Douarin et al., 1995), although the interaction is still ligand dependent (von Baur et al., 1996). Both TIF-1α and TIF-1β have been shown to interact with the Kruppel-associated box (KRAB) repression domain, a region highly conserved in proteins containing multiple Kruppel-type zinc fingers (Le Douarin et al., 1996; Moosmann et al., 1996). All these evidence about TIF-1 involvement in repression events complicates the interpretation of their role in a cellular context. Their mechanism of action is likely to involve chromatin rearrangement, but its nature still remains highly speculative (Le Douarin et al., 1996).

h. ARAs

The androgen receptor (AR) is of particular importance in prostate cancer: in the process of understanding its regulation, many AR specific coactivators have recently been identified. These androgen receptor activators (ARA), which are not a family of related factors, comprises ARA24 (Hsiao et al., 1999), ARA54 (Kang et al., 1999), ARA55 (Fujimoto et al., 1999) , ARA70 (Yeh and Chang, 1996) and ARA 160 (Hsiao and Chang, 1999). The only well-characterized member is ARA70 which was identified through its ligand-dependent interaction with the AF-2 of AR in a yeast two-hybrid screen. ARA70 has a particular ability to enhance the partial agonist activity of some AR hormonal antagonist in human prostate cancer cells DU145 (Miyamoto et al., 1998). Recently, ARA70's potential to coactivate PPARγ has open perspectives on its role in putative cross-talk between AR and PPARγ-mediated responses in cells (Heinlein et al., 1999).

i. TRIP-1 / SUG-1

SUG-1 was initially identified as a regulator of upstream activator sequences of the yeast activator Gal4 and was originally classified as a possible component of the RNA polymerase II holoenzyme (Xu et al., 1995). It is currently thought to be involved in the 2MDa yeast proteosome complex because it can be copurified with this complex and because a SUG-1 mutant in yeast shows reduced ubiquitin-dependent proteolysis (Rubin et al., 1996). Using TR and RXR in a yeast two-hybrid screen, Lee *at al.* identified an homologue of SUG-1 that they named TR interacting protein (TRIP-1) (Lee et al., 1995). Interestingly, this protein possesses a conserved ATPase domain but does not possess a consensus NR box motif.

j. Cointegrators: CBP and p300

Initially identified as a coactivator required for the activation of cAMP-response element binding protein (CREB) (Kwok et al., 1994), the CREB-binding protein (CBP) is now considered to act as a signal integrator for transcriptional regulation (Chakravarti et al., 1996; McKenna et al., 1999). CBP has been shown to act as a coactivator for many nuclear receptors such as ER, TR, RXR (Chakravarti et al., 1996; Kamei et al., 1996) and AR (Fronsdal et al., 1998). It also has a role as a coactivator for more general transcriptional regulators such as NF-κB (Perkins et al., 1997) and p53 (Avantaggiati et al., 1997). CBP also possesses a histone acetyl-transferase (HAT) activity (Bannister and Kouzarides, 1996) which can be regulated by the oncoprotein E1A and cyclin-dependent kinases (CDKs) (Ait-Si-Ali et al., 1998). This HAT activity has been shown to be active on substrates including the ACTR acetylase (Chen et al., 1999). In addition to its interactions with NRs, CBP also interacts with the SRC family of coactivators (Kamei et al., 1996; Torchia et al., 1997; Voegel et al., 1998). These interactions and the synergism of SRC-1 and CBP on ERα and PR transactivation (Smith et al., 1996) have lead to the hypothesis that CBP may form a complex with coactivators to potentate nuclear receptor activity. However, strong biochemical evidence tends to rule out the coexistence of SRC-1 and CBP in the same macromolecular complex (McKenna et al., 1998). This point is still a source of vivid interest.

p300 was identified independently from CBP, as a protein associated with the adenovirus E1A (Eckner et al., 1994). Its initial characterization suggested that the two proteins were similar and they were considered to form a family. Indeed, p300 has striking similarities with CBP: it enhances the same transcription factors and nuclear receptors, interacts with NRs in a ligand-dependent manner, interacts with the SRC family of coactivators and

possesses HAT activity. However, the genetic ablation of the p300 locus revealed that they are not completely functionally redundant as the knockout embryos showed major developmental and cell proliferation defects (Yao et al., 1998).

The current models of CBP/p300 action propose that they function as a common factor that "integrates" the signal arising from different cellular cues to regulate gene transcription (Janknecht and Hunter, 1996; Kamei et al., 1996). This regulation possibly occurs directly (through chromatin remodeling from their HAT function) and indirectly (through recruitment and activation of chromatin-remodeling protein complexes). A recent study on p300 action concluded it has no essential role in chromatin disruption itself but rather uses its acetyltransferase activity to stimulate TR-based transcription at a subsequent step (Li et al., 1999).

Some indication of the importance of CBP in regulating cells has come from the identification of a mutant associated with the human Rubinstein-Taybi syndrome, a disorder characterized by physical deformities and mental retardation (Petrij et al., 1995). More recently, CBP has also been shown to regulate promyelocytic oncogenic domains (POD), which are implicated in acute promyelocytic leukemia (Doucas et al., 1999).

5. Acetylation and deacetylation

Eukaryotic chromosomes are organized into a regularly repeating protein-DNA unit, the nucleosome. It is composed of 1.7 turns of a left-handed DNA superhelix wrapped around a highly basic protein complex, the histone octamer. These core particles contain two copies of each histones H2A, H2B, H3 and H4 (Richmond et al., 1984). Higher order of organization involves assembly of nucleosomes into chromatin domains via internucleosomal contacts mediated by histones amino-terminal tails (Luger et al., 1997).

The net effect of this compact arrangement is the inaccessibility of DNA substrate to general transcription factors. The catalytic transfer of acetyl groups to the amino-terminal lysine residues of histones H2A, H2B, H3 and H4 by histone acetylases (HATs), disrupts the interactions between nucleosomes and DNA, between neighboring nucleosomes, and possibly between nucleosomes and other proteins, resulting in a looser structure (Rhodes, 1997). This loss of compact structure facilitates access of transcriptional activators and coactivators to the promoter complex. Conversely, recruitment of histone deacetylases (HDs) results in the removal of the acetyl groups from histones amino-terminal lysine residues, resulting in a reestablishment of compact nucleosomal structure, restricting access to the promoter. It is the balance of these diametrically opposed activities of histone acetylation and deacetylation that is thought to regulate transcriptional activity/repression context at a given promoter. HAT activity has been initially associated with general transcription proteins such as HAT-A (Brownell et al., 1996) and $TAF_{II}250$ (Mizzen et al., 1996). Initial implication of the role of histones acetylation in nuclear receptor transcriptional regulation came from the identification of p/CAF intrinsic HAT activity (Yang et al., 1996). Then a vast number of coactivator were found to possess intrinsic HAT activity, including CBP itself (Bannister and Kouzarides, 1996; Ogryzko et al., 1996), hSRC-1 (Spencer et al., 1997), and hSRC-3 (Chen et al., 1997). Conversely, the activity of nuclear receptor corepressors such as SIN3 and NCoR has been linked to their capacity to interact with histone deacetylases HDAC-1 and HDAC-2 (Heinzel et al., 1997; Laherty et al., 1997), and to favor compaction of nucleosomes. Therefore, the presence and activity of some nuclear receptor coactivators/corepressors appears to be directly linked to the steady state of local chromatin compaction and subsequent level of gene transcription.

6. Goal of this study

The initial goal of this study was to identify proteins that would interact with nuclear receptors independently of their AF-2. After the initial identification of CIA by Dr. Linda D.B. McBroom (see details on page iv), the study became oriented toward the elucidation of CIA roles and partners. More specifically, I was interested in the identification of CIA interaction partners *in vitro* and *in vivo* and in the characterization of its mode of action in cells.

The work that consisted in the bulk of this thesis is presented in the form of a manuscript in chapter II. I have also included additional work that complements the data presented in the manuscript. This includes of our attemps to isolate a full-length cDNA clone for CIA (appendix A1), the identification of a putative physiological role for CIA in breast cancer cell lines (appendix A2) and finally, the rationale behind the selection of residues for mutagenesis in ERα based on previously published work (appendix A3).

CHAPTER II - MANUSCRIPT

The cloning and characterization of CIA: a novel nuclear receptor coactivator

This chapter forms the basis of the manuscript: "**CIA, a Novel Activation Function 2 (AF-2)-Independent Nuclear Receptor Coactivator**" by Frédéric Sauvé, Linda D. B. McBroom, Josette Gallant, Fernand Labrie and Vincent Giguère.

The role of each coautor in the preparation of this paper is described on page iv of this thesis.

ABSTRACT

Nuclear receptors coactivators are factors that enhance the receptor transcriptional activity. Most coactivators characterized to date exert their action through the receptor ligand-dependent activation function 2 (AF-2). This interaction is mediated by a coactivator LXXLL motif, and a hydrophobic cleft located within the terminal alpha helix of the receptor. In an effort to identify novel coactivators that function independently of AF-2, we used the LBD of the orphan receptor RVR (which lacks the terminal helix of the LBD) as a bait in a yeast two-hybrid screen. This strategy led to the cloning of a nuclear protein referred to as CIA (Coactivator Independent of AF-2 function) that shows wide tissue distribution. In addition to its interaction with RVR, CIA displayed specific interaction and coactivator activity with estrogen receptor (ER) α and β. The basal interaction of CIA with ERα and β is strongly enhanced by its natural ligand, 17β-estradiol (E_2), and surprisingly, also by the pure antiestrogens EM-800 and ICI 164,384. In contrast, tamoxifen and raloxifene blocked the E_2-induced CIA/ERα interaction, indicating that CIA may contribute to the distinct physiological actions of selective ER modulators (SERMs). While the E_2-dependent CIA/ERα interaction is mediated by the unique CIA LXXLL motif, the coactivator can interact with a AF-2 null mutant of ERα. In transfected cells, CIA does not affect RVR activity but enhances ERα transcriptional activity on both natural (pS2) and synthetic E_2-responsive promoters. Thus, CIA constitutes a novel type of ligand-dependent but AF-2-independent nuclear receptor coactivator that may play a specific role in ER physiology. Finally, the functional characteristics of CIA support a possible role for ligands in regulating the activity of orphan nuclear receptors lacking a AF-2 domain.

INTRODUCTION

Nuclear receptors (NR) belong to a superfamily of transcription factors that regulate hormonal processes involved in development, growth, metabolism and reproduction (Meyer et al., 1989). Family members can be classified into subgroups based on their dimerization potential, their ligand-binding specificity and their DNA-binding characteristics (Mangelsdorf and Evans, 1995; Mangelsdorf et al., 1995). Among members of the superfamily, there are a vast number of nuclear receptors for which no ligand has yet been identified. These receptors are classified as "orphans" and the identification of both their ligand and physiological role is of great interest as it could possibly uncover new hormone response systems (Giguère, 1999; Kliewer et al., 1999).

NR regulate gene transcription through two activation functions (Evans, 1988). The activation function 1 (AF-1) is ligand-independent and located in the N-terminus region of the receptor. The AF-1 serves as a docking site for coactivators and general transcription factors (Lanz et al., 1999; Lavinsky et al., 1998; Puigserver et al., 1998), and its activity can be regulated through post-translational modifications, such as phosphorylation (Hammer et al., 1999; Rochette-Egly et al., 1997; Tremblay et al., 1999). The second activation function (AF-2) is located within helix 12 of the well-conserved C-terminal ligand-binding domain (LBD) of the receptor (Danielian et al., 1992). The activation of AF-2 is dependent on ligand binding (Danielian et al., 1992; Meyer et al., 1989), which modifies the conformation of the LBD and promotes recruitment of coactivators (reviewed in Glass et al., 1997). Both activation functions are required for maximal transcriptional activity of the receptor.

The coactivators recruited to the receptor affect its transcriptional activity through multiple mechanisms (reviewed in McKenna et al., 1999). Some form bridges with the basal transcription machinery (Schulman et al., 1995) or act as recruitment partners for other cofactors, principally for the p300/CBP "cointegrators" (Chakravarti et al., 1996).

Others, like the steroid receptor coactivator-1 (SRC-1) family, possess multiple activation domains which cooperate to maximize receptor activity (Oñate et al., 1998). Some coactivators have been shown to possess protein kinase activity and participate in phosphorylation events necessary for full activity of the transcription complex (Fraser et al., 1998). Finally, others show a variety of alternative activities, such as TIF-1 which interacts with a putative chromatin remodeling complex (Le Douarin et al., 1995) and P/CAF and SRC family members which possess intrinsic acetyltransferase activity (Spencer et al., 1997; Yang et al., 1996). All these coactivators are thought to exert their action as part of a large complex that participates actively in transcription, possibly through modification of the chromatin template.

The ligand-dependent interaction between coactivators and nuclear receptors is mediated by a motif consisting of the sequence LXXLL (where L is leucine and X is any amino acid) which has been called an NR-box (Darimont et al., 1998; Ding et al., 1998; Heery et al., 1997) as well as an LXD domain (McInerney et al., 1998). The specificity of interaction of a given coactivator with nuclear receptors depends on the number and the spacing between each LXXLL motif (McInerney et al., 1998). NR-boxes interact with a hydrophobic cleft, a region which is located on the surface of the LBD of nuclear receptors (Feng et al., 1998). The hydrophobic cleft is formed from helices 3, 5, 6 and 12, residues contained in these helices make direct contact with the LXXLL motif. This region becomes fully functional only when the cognate ligand binds the LBD, an event that repositions helix 12 and results in the formation of the complete interaction surface. The ligand-dependent interaction of coactivators with the LBD of NRs can be abolished by antagonist-induced disruption of the position of helix 12 (Brzozowski et al., 1997; Shiau et al., 1998). In addition, specific mutations of helix 12 can also impair coactivator interaction with the LBD (Collingwood et al., 1998; Kamei et al., 1996; Saatcioglu et al., 1993).

In an effort to identify novel coactivators that function independently of AF-2, we performed a yeast two-hybrid screen using the LBD of RVR (Retnakaran et al., 1994) (NR1D1), an orphan nuclear receptor lacking an AF-2. We report the identification of a novel coactivator whose interaction with nuclear receptors is ligand-dependent but independent of AF-2 integrity.

MATERIALS AND METHODS

CIA isolation and interaction assay in yeast. *Saccharomyces cerevisiae* Y190 {MATa gal4 gal180 his3 trp1 901 ade2-101 ura3-52 leu2-3,-112 + URA3xGAL→lacZ, LYS2::GAL(UAS)→HIS3 cys} containing a bait plasmid pAS1-RVR (aa286-509) was transformed with a human fetal kidney library (19-23 weeks) and plated on a SD medium (MATCHMAKER Library Protocol, Clontech) lacking tryptophan, leucine and histidine and containing 50 mM 3-aminotriazole. His^+ colonies having β-galactosidase activity, as determined by a filter lift assay, were further characterized via standard techniques (Bartel et al., 1993). The library plasmids were recovered by isolating total yeast DNA, electroporated into *E. coli* HB101, and isolated on a minimal medium lacking leucine and containing ampicillin. For the interaction assays, CIA was retransformed into Y190 and mated to Y187 strain containing various baits (SNF1, Lamin, CDK1, p53, hERRα (NR3B1), rERRβ (NR3B2), hRevErbα (NR1D1), mRVR (NR1D2), hGRα (NR3C1) and hRARα (NR1B1)). The baits were assayed for interaction with pGAD-CIA as described elsewhere (Lee et al., 1995).

Plasmid constructs and reagents. To construct the yeast-two hybrid bait, pCMX-RVR (described in ref Retnakaran et al., 1994) was digested with *Bst*XI, end- filled with Klenow and digested *Bam*HI. The 1.2 kb fragment containing the LBD was subcloned into pAS1 digested with *Nco*I, end filled with Klenow and digested with *Bam*HI. pCMX-

CIA was constructed by PCR using a forward oligo introducing a consensus start site (underlined): 5'-ACGGAATTCGTACCATGGCGCCTTTGTCCTACGGC-3' and a reverse oligo: 5'-GCGCGAATTCTCAGTAATGCCTCTGGTA-3'. The PCR product was digested with *Eco*RI and cloned into pCMX. A plasmid expressing the GFP-CIA fusion protein was constructed as follows: pCMX-CIA was digested with *Eco*RI and CIA-ORF was cloned into pEGFP-C2 (Clontech) digested with *Eco*RI. GST-RVR was constructed by cutting pCMX-RVR with *Nco*I and *Bam*HI, end-filling with Klenow and cloning the insert into *Sma*I cut pGEX-2T vector (Pharmacia Biotech). All the GST-RVR LBD deletions were constructed as follows: PCR using specific 5' and 3' oligos was performed and the products were digested with *Bam*HI and *Mfe*I. The fragments were cloned between the *Bam*HI and *Eco*RI sites of the pGEX-2T vector. GST-CIA was constructed as follows: pCMX-CIA was digested with *Bam*HI and *Asp*718I, end filled with Klenow and the 1030 bp fragment was cloned into the *Sma*I site of pGEX-3X (Pharmacia Biotech). pCMX, pCMX-hERα, TKLuc, 2C-vERE-TKLuc, PS2-Luc and PS2(ΔERE)-Luc reporters have been described previously (Tremblay et al., 1997) as has the MTV-Luc reporter (Hollenberg and Evans, 1988). 3C-TREPal-TKLuc was constructed by cloning 3 copies of a TREpal (Umesono et al., 1988) consensus oligo between the *Hind*III and *Bam*HI sites of TKLuc. All the ERα and the CIA mutants were constructed by PCR site-directed mutagenesis using *Pfu* polymerase (Stratagene) and the smallest possible fragment containing the mutation(s) was sequenced, cut out and reinserted into the template plasmid to eliminate the risk of undesired mutations. The framing of all constructs described here was confirmed by DNA sequencing. SRC-1 was a gift of Joe Torchia, University of Western Ontario, London, Ontario, Canada. Estradiol (E_2), all-*trans* retinoic acid, 3-iodothyroxine, progesterone and dexamethasone were all obtained from Sigma Chemical Co. (St. Louis, Mo.). 1,25-dihydroxyvitamin D was a generous gift of Alain Moreau, Institut de Recherches Cliniques de Montréal, Montréal, Québec, Canada. EM-652 and ICI 182,780 were synthesized in the medicinal chemistry

division of the Laboratory of Molecular Endocrinology, CHUL research Center, Québec, Québec, Canada. OHT was kindly provided by D. Salin-Drouin, Besins-Iscovesco, Paris, France.

Protein expression. The various bait protein constructs were transformed either in *E. coli* DH5α (GST-RVR LBD) or BL21(DE3)pLysE (GST-CIA) and protein expression was induced with 0.4 mM isopropylthiogalactopyranoside (IPTG) at 37°C for 3 h (0.05 mM IPTG in the case of GST-CIA{LXXAA}). Bacterial extracts were prepared via sonication (DH5α) or freeze-thaw cycles (BL21(DE3)pLysE). The extracts were aliquoted, freezed in an ethanol/dry ice bath and storeded at -80°C.

Cell Culture and Transfection. COS-1 and HeLa cell lines were obtained from the American Type Culture Collection. Cells were routinely cultured in Dulbecco's Minimal Essential Medium (DMEM) containing penicillin (25 U/mL), streptomycin (25 U/mL) and 10% fetal calf serum (FCS) at 37°C with 5% CO_2. Twenty-four hours prior to transfection, the cells were split and seeded into 12 well-dishes. At this stage, the media was changed for phenol-red free DMEM supplemented with antibiotics and 10% charcoal-dextran treated FCS. Cells were transfected using the calcium phosphate-DNA coprecipitation method (Giguère et al., 1990). Typically, 0.5 μg of reporter plasmid, 0.2 μg of internal control (CMV-βGal), 50 ng of receptor expression vector, 100 ng of coactivator expression vector and carrier (Bluescript pKS II) to a total of 1 μg per well were added to the cells. After 12-14 h, cells were washed twice with PBS and treated with either 10^{-8} E_2 or carrier (ethanol) for 24 h in phenol red-free DMEM supplemented with 10% stripped FCS. Cells were then washed and harvested in a potassium phosphate lysis buffer containing 1% Triton X-100. Luciferase and β-galactosidase assays were performed as previously described (Tini et al., 1993). All the transfection results presented are the average of at least two independent experiments performed in triplicate.

The GFP/CIA-GFP intracellular localization experiments were conducted as follows: 24 prior to transfection, HeLa cells were seeded into 6-wells dishes with DMEM supplemented with 10% FCS. GFP or CIA-GFP were transiently transfected at 2 μg per well using calcium phosphate-DNA coprecipitation. After transfection, the cells were washed twice with PBS and recuperated in media. Pictures were taken using a CCD camera mounted on a Zeiss Axiovent-135 microscope. Image capture and analysis was performed via Northern Eclipe software (EMPIX, Mississauga, Canada).

Northern Blots. Total RNA was collected from different tissues of 17.5 dpc mouse embryos and processed with TRIZOL reagent (Life Technologies). Poly-A^+ RNA was prepared using QuickPrep Micro mRNA purification kit (Pharmacia Biotech). The samples were separated by electrophoresis in a 1% agarose-0.4% formaldehyde-1x MOPS (morpholinepropanesulfonic acid) gel and transferred onto a nylon membrane (Hybond N; Amersham life sciences) in 20x SSC buffer (1x SSC is 0.15M NaCl and 0.015M Na_3citrate at pH7.0). The RNA was UV cross-linked to the membrane and then prehybridized at 42°C for 1 h in a buffer containing 50% formamide, 5x SSPE (1x SSPE is 0.15M NaCl, 0.01M NaH_2PO_4 and 1mM EDTA at pH7.4), 5x Denhardt's solution, 1% glycine and 100 μg/ml of denatured salmon sperm DNA. After prehybridation, the membranes were hybridized overnight at 42°C in a solution of 50% formamide, 5x SSPE, 1x Denhardt's solution, 0.3% SDS, 10% (wt/vol) dextran sulfate, 100 μg/ml of denatured salmon sperm DNA and $6x10^5$ cpm/ml of ^{32}P-labeled probe. Membranes were then washed as following: 2x SSC 1% SDS at 55°C for 20 min, 0.2x SSC 0.1% SDS for 20 min at 55°C and for another 20 min at 65°C. They were then autoradiographed at –70°C on a X-OMAT film (Eastman Kodak Company) and/or quantified using phosphorimager (Fuji Photo Film Inc.).

GST Pull-down assays. Fusion proteins were incubated with glutathione-Sepharose beads (Pharmacia Biotech) for 20 min at 4°C. The beads were then spun down, washed 4 times with GST-binding buffer (20 mM HEPES pH 7.9, 150 mM KCl, 5 mM $MgCl_2$, 0.1% 3-{(3-Cholamidopropyl)dimethyl-ammonio}-1-propanesulfonate (CHAPS), 0.1 mM PMSF and 1 μM leupeptin). The beads were then resuspended in 150 μl of GST-binding buffer with 20 μg ml^{-1} BSA, the appropriate hormone (or carrier) and 5 μl of *in vitro* translated protein prepared using rabbit reticulocyte lysates (Promega, Madison, Wi.). The reactions were incubated 90 min at 4°C with mild agitation. The complexes were then spun down and washed in GST-binding buffer, twice with 20 μg/ml ml BSA and twice without BSA. Samples were then resuspended in 2x SDS-sample buffer and boiled for 5 min prior to separation on 10% SDS-PAGE. Gels were then fixed and treated with the fluorographic reagent Amplify (Amersham Life Science), dried and exposed at –80°C. To ensure the presence of equal amount of bait proteins, purified extracts from the various baits were previously separated on SDS-PAGE, stained with coomassie blue and compared. Equivalent amount of bait proteins were then used for each pull-down experiment.

RESULTS

Initial cloning of CIA. In an effort to identify novel coregulatory proteins that would act independently of AF-2, we used the LBD of RVR, an orphan nuclear receptor with no AF-2 (Retnakaran et al., 1994) as a bait in a yeast two-hybrid screen. Standard procedure was followed (Chien et al., 1991) and a putative cDNA clone was obtained from a human fetal kidney expression library. Sequencing of this 2155 base pair (bp) clone revealed a 623 amino acid (AA) long open reading frame (ORF) (Fig. 1A). Sequence comparison searches failed to reveal significant homology with known proteins. Only two identifiable features present in this ORF: an NR-box at AA 387-391 (boxed in Fig. 1A) and an amino terminal arginine and aspartic acid-rich region (RD-rich region, underlined in Fig. 1A). A schematic representation of the protein features is presented in Fig. 1B. Extensive screening and rapid amplification of cDNA ends (RACE) efforts did not generate a clone with a longer 5'-end. (as described in appendix A1)

The specificity of interaction of the clone was verified in yeast through liquid β-galactosidase assay by testing with two panels of baits fused to Gal4 DBD. First, we assessed its interaction with a panel of non-specific baits (Fig. 2A). In this test, the clone failed to interact with SNF1, lamin, CDK1 and p53 but interacted strongly with RVR. These results confirm the specificity of interaction for the bait with a variety of unrelated proteins. In a second panel, we measured its potential to interact with different nuclear receptors in the absence of ligand (Fig. 2B). In this assay, the clone displayed strong interaction with RVR and also with Rev-ErbAα (NR1D1) (another closely related orphan receptor lacking the AF-2 domain): no interaction was observed with hERRα (NR3B1), rERRβ (NR3B2), hERα (NR3A1), hGRα (NR3C1) and hRARα (NR1B1). On the basis of its specificity of interaction for orphans lacking an AF-2 and other characteristics that will be discussed below, we named this clone CIA (Coactivator Independent of AF-2).

Tissue expression and cellular localization of CIA. In order to determine if CIA expression exhibits tissue specificity, we performed northern blots using poly-A^+ mRNA extracted from various tissues of fetal (e17.5) mouse: (Fig. 3a, upper panel) expression of mouse CIA mRNA was observed in all tested tissues. The level is very low in embryonic liver but high in heart and kidney. Note the presence of two transcripts in the embryonic kidney (lane 5). The difference between the two transcripts, about 700pb, is due to use of an alternative poly-adenylation signal, which can be found on expressed sequence tag (EST) clones (discussed in appendix A1). mRNA integrity was verified by probing with β-actin (Fig. 3a, lower panel) and equal loading was ensured via ethidium bromide staining (data not shown).

To assess the intracellular localization of CIA, transient transfection experiments were performed in HeLa cells using a fusion of the green fluorescent protein (GFP) with the ORF of CIA. GFP alone distributes evenly throughout the cell (Fig. 3C and D), whereas CIA-GFP fusion proteins are strictly restricted to the nucleus (Fig. 3E and F). The same results were obtained when the experiments were performed in COS-1 cells (data not shown). Therefore, the CIA-GFP fusion protein is strictly nuclear when transfected into mammalian cells.

***In vitro* interaction of CIA with nuclear receptors.** To confirm the interaction of CIA with RVR previously observed in yeast, *in vitro* pull-down experiments were performed. As expected from the screening interaction, *in vitro* translated CIA interacts with both GST-full length and GST-LBD constructs of RVR (Fig. 4A). To test whether the interaction was specific to RVR or if CIA interacts with other nuclear receptors, a GST-fusion containing amino acids 55 to 395 of CIA was constructed and pull-down experiments were performed using *in vitro* translated nuclear receptors (Fig. 4B). CIA did not interact with members of the non-steroid nuclear receptors: triiodothyronine receptor α ($T_3R\alpha$), all-*trans* retinoic acid receptor α (RARα) and 1,25-dihydroxyvitamin D

receptor (VDR). It also did not interact with the steroid nuclear receptors GR and PR. No interaction could be detected between CIA and the following nuclear receptors either in the presence or absence of ligand: 9-*cis* retinoic acid receptor β (RXRβ), triiodothyronine receptor β (T_3Rβ), retinoic acid-related receptor α (RORα) (NR1F1) and the mineralocorticoid receptor (MR) (data not shown). Strikingly, however, CIA interacted with ERα and in a weaker fashion with ERβ (data not shown), and this interaction was greatly enhanced in the presence of E_2. Therefore, CIA shows *in vitro* specificity of interaction for ERα and β and the orphan nuclear receptors RVR and Rev-erbAα.

To explore the possibility that CIA acts as a coactivator *in vivo*, transient transfection experiments were performed in COS-1 cells (Fig. 5). When cotransfected with NRs in presence of ligand, CIA potentiated ERα transcriptional activity on a consensus vitellogenin response element (vERE) reporter but did not alter T_3Rα or PR transcription on their respective consensus element (Fig. 5A). These results confirmed the specificity of CIA for ER that was observed *in vitro*. CIA also enhanced ERα transactivation on the pS2 natural promoter: this effect required the presence of an intact estrogen response element (ERE) (Fig. 5B). In addition, the effect of CIA on ER transactivation was slightly greater on the PS2 element than on the vERE. To further support the promoter independent effect of CIA on ER, we constructed a chimera of the ERα in which the C-terminal extremity of the receptor was fused to the DNA-binding domain (DBD) of the yeast Gal4 transcription factor. When the transactivation potential of this fusion protein was assessed on a upstream activator sequence (UAS) reporter construct, the CIA effect was similar to what was obtained on a vERE element (data not shown). These experiments were also performed in HeLa cells and yielded similar results, showing that the observed effects were cell-type independent (data not shown).

CIA interaction with NR is dependent on the conserved signature motif. To determine which region of the LBD of RVR interacts with CIA, we performed *in vitro*

pull-down experiments with various deletions of RVR LBD (Fig. 6A). The regions necessary for the interaction are located around helices 3 and 11 (observe lanes 5 and 8). In addition, mutation of key residues in the well conserved signature motif (FAK to AGA at amino acids 415-417 and PGF to AGA at amino acids 420-422) markedly reduced the interaction (lanes 10 and 11). This experiment identified helices 3 and 11, as well as the signature motif as regions necessary for the interaction between CIA and RVR.

To evaluate CIA interaction with ER, we constructed N-terminal and C-terminal truncations of ERα and tested their ability to interact with CIA *in vitro* (Fig. 6B). Ligand-dependent interaction of CIA with the C-terminal construct of ERα (CDEF) was even more potent then with the wild type receptor (compare lanes 7 and 9 to lanes 3 and 4). Conversely, the N-terminal construct of ERα (ABC) did not interact with CIA. These results show that CIA interaction with ERα occurs through the C-terminal extremity of the receptor, similarly to RVR. In order to identify the exact residues implicated in the ligand-dependent interaction between CIA and ERα, we based our initial approach on the RVR interaction data and constructed mutants in the signature motif of ERα. Mutations of lysine 362 and the entire consensus WAK (aa360-362) were made and subsequently tested for their ability to interact with CIA using *in vitro* pull-down experiments (Fig. 6C). Both signature motif mutants abolished the ligand-dependent interaction with ERα (compare lanes 6 and 9 to lane 3). These results show that the interaction between CIA and ERα requires the integrity of the signature motif and especially of lysine 362.

To further characterize the interaction of CIA with ERα, a panel of antagonists were used to test whether the ligand-dependent interaction could be modulated (Fig. 6D). In a pull-down experiment, *in vitro* translated ERα ligand-dependent interaction with GST-CIA can be significantly reduced by addition of the partial antagonists 4-hydroxytamoxifen (OHT) (lanes 5 and 6) or raloxifene (lanes 7 and 8). The E_2-induced interaction was abolished (compare lanes 6 and 8 to lane 4) while the basal interaction was not affected (compare lanes 5 and 7 to lane 3). Remarkably, "pure" antiestrogens

such as ICI 182,780 (lanes 9 and 10) and EM-652 (lanes 11 and 12) were unable to block the E_2-induced interaction (compare lanes 10 and 12 to lane 4): in fact, their presence increased the interaction of ERα with CIA (compare lanes 9 and 11 to lane 3).

Ligand-dependent interaction occurs through an NR-box. Since NR-boxes have been shown to be the region of coactivators that mediate ligand-dependent interaction with NRs, we next tested if CIA interaction with ERα and β was mediated by this motif. Mutagenesis of the only NR-box consensus motif in CIA, the LINLL (amino acids 387-391) into LINAA, was performed and the ability of this mutant to interact with the estrogen receptors was tested *in vitro* (Fig. 7A). The mutation of both leucine residues to alanine abolished the ability of CIA to interact with both the ERα and β in a ligand-dependent manner (compare lane 6 and lane 4). To confirm *in vivo* that the potential of the CIA mutant to coactivate ERα would be reduced, we tested its ability to coactivate ERα in transient transfections (Fig. 7B). As expected, the mutated CIA is less effective at enhancing ERα transactivation than the wild type CIA.

Interaction of CIA with ERα is independent of AF-2. In order to further characterize CIA interaction with the LBD surface of the estrogen receptors, we mutated residues located in the previously defined hydrophobic cleft on the surface of the LBD (Feng et al., 1998). These residues have previously been shown to either greatly reduce (V376R) or completely abolish (E542K) ligand-dependent interaction with coactivators (Feng et al., 1998). We also studied an ERα AF-2 null mutant (L539A) that has previously been shown not to interact in a ligand-dependent fashion with SRC-1 (Tremblay et al., 1998; White et al., 1997). The rationale underlying the choice of these receptor mutants is outlined in appendix A3. *In vitro* pull-down experiments were performed with the various ERα mutants and their ligand-dependent interactions with CIA were compared to that of the wild type ERα (Fig. 8A). The mutant V376R shows

decreased ligand-dependent interaction with CIA. This observation mimics what was previously demonstrated with other coactivators (Feng et al., 1998) and suggests that this residue participates in the interaction with CIA. Strikingly, both E542K and L539A mutants still show ligand-dependent interaction with CIA. This result is different from what was previously reported for other coactivators and indicates that the integrity of these residues is not required for the interaction with CIA. Both L539 and G542 are part of the helix 12 of the LBD, forming the core of the AF-2. The fact that these residues are not necessary for CIA interaction with ERα confirms that it occurs through a ligand-dependent but AF-2 independent mechanism. To confirm this result *in vivo*, transient transfections were performed using the AF-2 L539A null mutant of ERα (Fig. 8B). When this mutant was cotransfected with SRC-1, no ligand-dependent transactivation could be restored. In contrast, when the ERα AF-2 mutant was cotransfected with CIA, ligand-dependent transactivation activity could be observed.

DISCUSSION

CIA sequence and features. Analysis of CIA sequence reveals very little information about its putative role: (Fig. 1B) the only identifiable motifs contained in CIA are an NR-box and a RD-rich region. The NR-box motifs have extensively been shown to mediate ligand-dependent interaction with nuclear receptors (Darimont et al., 1998), but to our knowledge, RD-rich regions have not been linked conclusively with any protein function. The RD cluster represents a highly hydrophilic region of alternating positively and negatively charged residues, which are most likely exposed at the surface of the protein. The coactivator TRAP220 (Treuter et al., 1999), also contains an RD-rich region, which is only 14 amino acids long and was suggested to be involved either in the oligomerization of TRAP220 or in DNA-binding. It is possible that the RD cluster plays a similar role in CIA, although the significantly longer RD-rich domain may play a different role in this novel protein. We have tested if this region could bind specifically to DNA using standard DNA-binding assays and no conclusive interaction has been observed (data not shown).

CIA specificity of interaction for the RVR and β and the ERs. Of all the nuclear receptors tested, only RVR and Rev-erbAα and ERα and β were able to interact with CIA. This result may be related to the fact that CIA possesses a single NR-box, and coactivators possessing only one of these motifs have been shown to be less promiscuous with putative partners (McInerney et al., 1998). Specifically, ERα was shown to be able to interact with coactivators possessing only one NR-box while others receptors like T_3R, RAR, PPAR and PR interact only with coactivators containing two of these motifs with an appropriate spacing (McInerney et al., 1998). Based on our interaction data, we propose that the RVR and RevErbα orphan receptors will interact with coactivators

possessing only one NR-box. This information can be of great interest in order to establish a coactivator interaction screen to identify putative ligands for these orphans.

Our transfection data supports CIA as being a coactivator of ERα transcriptional activity. This enhancement is more pronounced on a natural promoter (pS2) than on an artificial one (vERE). This effect is also cell-type independent, suggesting that *in vivo*, CIA can act in multiple cellular types and contexts, in agreement with the wide distribution of its mRNA. CIA will affect ER activity on promoters containing functional ERE and could potentially compete or cooperate with other coactivators. CIA may act as part of a coactivator complex containing multiple factors that could help stabilize interactions with other proteins. Current assays may not be suitable to analyze the full extent of CIA contribution in cellular transcription regulation. The possibility also exists that CIA acts as a bridging protein, recruiting factors in a transcription complex. Although, at this point, there is no evidence to support this hypothesis, the absence of conserved structural functions in CIA points to either a new and previously unreported mode of action or a more structural role for the protein.

The effect of CIA on RVR transcriptional effect is harder to observe and interpret due to the nature of the available model systems. Off the few characterized roles of RVR, its ability to repress RORα transcriptional activity through direct competition for a RORE binding site, on the N-*myc* promoter (Dussault and Giguère, 1997), appeared to be the most appropriate to evaluate the role of CIA. Unfortunately, cotransfection of CIA together with RVR and RORα (on a RORE element) did not induce a significant relief of RVR repression mediated by synthetic RORE-containing reporter plasmids (data not shown). Also, the co-transfection of CIA and RVR did not yield any significant transcriptional activation (data not shown). Those two experiments suggest a role for an unidentified RVR ligand that could be necessary to detect an eventual CIA effect on its transcriptional activity. Therefore, CIA could prove to be an important tool for the

identification of ligands for members of the Rev-ErbA family of orphan nuclear receptors.

CIA interaction does not require AF-2 integrity. It was previously established (Feng et al., 1998) that the hormone-dependent SRC family of coactivators bind to the "hydrophobic cleft" on the surface of NR. This ligand-dependent interaction requires an intact AF-2. CIA, although clearly not a member of the SRC family, interacts in a ligand-dependent fashion with NRs through an NR-box. The fact that CIA interaction does not require an intact AF-2 -since it still interacts with ERα L539A and E542K mutants- suggests that its identification has uncovered a novel type of AF-2 independent coactivator. The interaction of CIA with ER cannot be blocked by "pure" antagonists such as EM652 and ICI 187,780, which are believed to prevent the binding of coactivators by disrupting the positioning of helix-12 on the surface of the LBD. The fact that CIA still interacts with ER in the presence of these "pure" antagonists confirms that helix 12 integrity is not required for its interaction. Therefore, CIA represents a putative novel class of ligand-dependent coactivators that mediate their action independently of AF-2 integrity. Identification and characterization of such coactivators is important in understanding nuclear receptor transcriptional regulation. Better comprehension of this regulation will help us develop a global scheme of NRs action in mediating endocrine defects and could ultimately be used to design appropriate therapeutics.

FIG. 1. Sequence and features of the CIA cDNA clone. (A) The sequence obtained from the human fetal kidney cDNA is 2155 bp long and contains a 621 amino acid long open reading frame. The arginine and aspartic acid rich region is underlined and the LINLL NR-box interaction motif is boxed. The putative translational initiation site is in bold. Nucleotides are numbered on the right. (B) Schematic representation of the protein with the features highlighted.

A

```
GCGCCTTTGTCCTACGGCGGGCAGGTGGGCCGACGCGGAGGCGGCAGCGGCGGGCCTGAGGCGAAGGAGCGGCCGGGAGCCCGCCGCGCT   90
 A  P  L  S  Y  G  G  Q  V  G  R  R  G  G  G  S  G  G  P  E  A  K  E  R  P  G  A  R  R  A

GGTAGCGATATTAATAAGGCAGCGGAAAGAAGAAATATGAATACGGCTCCATCAAGACCCAGCCCCACACGAAGGGATCCATATGGCTTT  180
 G  S  D  I  N  K  A  A  E  R  R  N  M  N  T  A  P  S  R  P  S  P  T  R  R  D  P  Y  G  F

GGAGACAGTCGAGATTCAAGGCGTGATCGATCCCCAATTCGAGGAAGTCCAAGGAGAGAGCCCAGGGATGGCAGAAATGGCCGGGATGCC  270
 G  D  S  R  D  S  R  R  D  R  S  P  I  R  G  S  P  R  R  E  P  R  D  G  R  N  G  R  D  A

CGGGACAGCAGAGACATTCGAGACCCCCGAGACTTGCGGGACCACAGACATAGTAGAGATTTGCGGGATCACAGAGACAGCAGGAGTGTG  360
 R  D  S  R  D  I  R  D  P  R  D  L  R  D  H  R  H  S  R  D  L  R  D  H  R  D  S  R  S  V

CGCGACGTTCGGGACGTGAGGGATCTTAGAGACTTTCGTGATCTAAGAGACTCTAGGGATTTTCGAGATCAGCGAGACCCCATGTACGAC  450
 R  D  V  R  D  V  R  D  L  R  D  F  R  D  L  R  D  S  R  D  F  R  D  Q  R  D  P  M  Y  D

AGATACAGAGACATGAGAGACTCCCGAGATCCTATGTACAGGAGAGAAGGCTCTTATGACCGATACCTACGAATGGATGACTATTGCAGG  540
 R  Y  R  D  M  R  D  S  R  D  P  M  Y  R  R  E  G  S  Y  D  R  Y  L  R  M  D  D  Y  C  R

AGAAAGGATGACTCTTATTTTGACCGTTACAGAGATAGCTTTGATGGACGGGGCCCTCCAGGCCCAGAAAGTCAGTCTCGTGCAAAAGAG  630
 R  K  D  D  S  Y  F  D  R  Y  R  D  S  F  D  G  R  G  P  P  G  P  E  S  Q  S  R  A  K  E

CGTTTGAAACGTGAGGAACGGCGTAGAGAAGAGCTTTATCGTCAATATTTTGAGGAAATCCAGAGACGCTTTGATGCCGAAAGGCCCGTT  720
 R  L  K  R  E  E  R  R  R  E  E  L  Y  R  Q  Y  F  E  E  I  Q  R  R  F  D  A  E  R  P  V

GATTGTTCTGTGATTGTGGTCAACAAACAGACAAAAGACTATGCTGAGTCTGTGGGGCGGAAGGTGCGAGACCTGGGCATGGTAGTGGAC  810
 D  C  S  V  I  V  V  N  K  Q  T  K  D  Y  A  E  S  V  G  R  K  V  R  D  L  G  M  V  V  D

TTGATCTTCCTTAACACAGAAGTGTCACTGTCACAAGCCTTGGAGGATGTTAGCAGGGGAGGTTCTCCTTTTGCTATTGTCATCACCCAG  900
 L  I  F  L  N  T  E  V  S  L  S  Q  A  L  E  D  V  S  R  G  G  S  P  F  A  I  V  I  T  Q

CAACACCAGATTCACCGCTCCTGCACAGTCAACATCATGTTTGGAACCCCGCAAGAGCATCGCAACATGCCCCAAGCAGATGCCATGGTG  990
 Q  H  Q  I  H  R  S  C  T  V  N  I  M  F  G  T  P  Q  E  H  R  N  M  P  Q  A  D  A  M  V

CTGGTGGCCAGAAATTATGAGCGTTACAAGAATGAGTGCCGGGAGAAGGAACGTGAGGAGATTGCCAGACAGGCAGCCAAGATGGCCGAT 1080
 L  V  A  R  N  Y  E  R  Y  K  N  E  C  R  E  K  E  R  E  E  I  A  R  Q  A  A  K  M  A  D

GAAGCCATCCTGCAGGAAAGAGAGAGAGGAGGCCCTGAGGAGGGAGTGCGTGGGGGCCACCCTCCAGCCATCCAGAGCCTCATCAACCTG 1170
 E  A  I  L  Q  E  R  E  R  G  G  P  E  E  G  V  R  G  G  H  P  P  A  I  Q  S  L  I  N  L

CTGGCAGACAACAGGTACCTCACTGCTGAAGAGACTGACAAGATCATCAACTACCTGCGAGAGCGGAAGGAGCGGCTGATGAGGAGCAGC 1260
 L  A  D  N  R  Y  L  T  A  E  E  T  D  K  I  I  N  Y  L  R  E  R  K  E  R  L  M  R  S  S

ACCGACTCTCTGCCTGGCCCGATTTCCCGCCAACCACTCGGGGCGACCTCGGGTGCCTCGCTGAAGACACAGCCAAGCTCCCAACCGCTC 1350
 T  D  S  L  P  G  P  I  S  R  Q  P  L  G  A  T  S  G  A  S  L  K  T  Q  P  S  S  Q  P  L

CAGAGCGGCCAAGTGCTCCCCTCTGCTACACCCACTCCATCTGCACCCCCCACCTCCCAGCAAGAGCTTCAGGCCAAAATCCTCAGCCTC 1440
 Q  S  G  Q  V  L  P  S  A  T  P  T  P  S  A  P  P  T  S  Q  Q  E  L  Q  A  K  I  L  S  L

TTCAATAGTGGCACAGTGACGGCCAATAGCAGCTCTGCATCCCCCTCGGTTGCTGCCGGAAACACCCCAAACCAGAATTTTTCCACAGCA 1530
 F  N  S  G  T  V  T  A  N  S  S  S  A  S  P  S  V  A  A  G  N  T  P  N  Q  N  F  S  T  A

GCAAACAGCCAGCCTCAACAAAGATCACAGGCTTCTGGCAATCAGCCTCCAAGCATTTTGGGACAGGGAGGATCTGCTCAGAACATGGGC 1620
 A  N  S  Q  P  Q  Q  R  S  Q  A  S  G  N  Q  P  P  S  I  L  G  Q  G  G  S  A  Q  N  M  G

CCCAGACCTGGGGCTCCTTCCCAAGGGCTTTTTGGCCAGCCTTCCAGTCGCCTGGCACCTGCTAGCAACATGACTAGCCAGAGGCCTGTG 1710
 P  R  P  G  A  P  S  Q  G  L  F  G  Q  P  S  S  R  L  A  P  A  S  N  M  T  S  Q  R  P  V

TCTTCCACAGGTATCAACTTTGACAATCCAAGTGTACAGAAGGCTCTGGATACCCTGATCCAGAGTGGCCCTGCTCTCTCCCACCTGGTT 1800
 S  S  T  G  I  N  F  D  N  P  S  V  Q  K  A  L  D  T  L  I  Q  S  G  P  A  L  S  H  L  V

AGCCAGACCACAGCACAGATGGGGCAGCCACAGGCCCCCATGGGATCTTACCAGAGGCATTACTGAAGCTAAATCTTTCAACTCTCCCCA 1890
 S  Q  T  T  A  Q  M  G  Q  P  Q  A  P  M  G  S  Y  Q  R  H  *

GTCCCCTCTCCCCTGGCCTCCTCCCACTTACTTGTTCTAAATAGAGCTGTTTGAGATGTTCTCTGCGCTCCCAGGCCGGCATCGAGTGTC 1980
ATCAATTTCTACCACCTGCTCTCTCTTCTGCCCAAGGCTGTGTTGCTTATTCCTTACAAAGTTTATACTGCATTTGGGGCTGTATCTTTT 2070
TTTGTTTTGTTTTTTTTTTTTCCTCTTTGGAACTCTTGTGTTGTTAATAAAATGAAATGATTACTTTTTAATTAAAATGTCGAC       2155
```

B

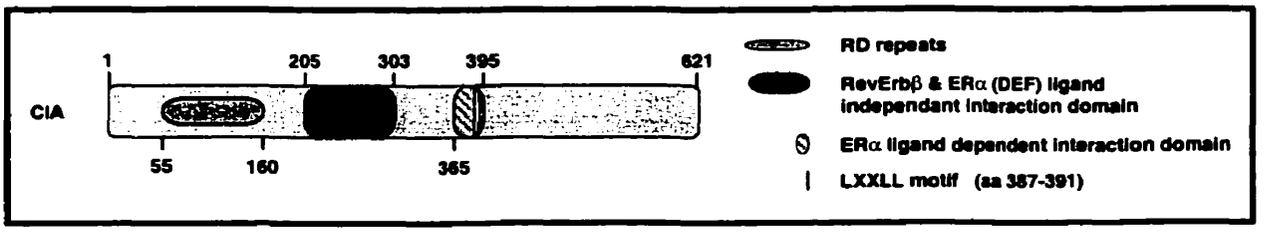


FIG 2. Specificity of nuclear receptor-CIA interactions in yeast. (A) Liquid β-galactosidase assays demonstrate that CIA interacts with RVR but not with control plasmid pAS1 nor with unrelated baits SNF1, lamin, CDK1 and p53. (B) In a similar assay, CIA interacts only with Rev-ErbAα and RVR, and does not interact with the control plasmid nor with the nuclear receptors hERRα, rERRβ, hERα, hGRα and hRARα in the absence of ligands.

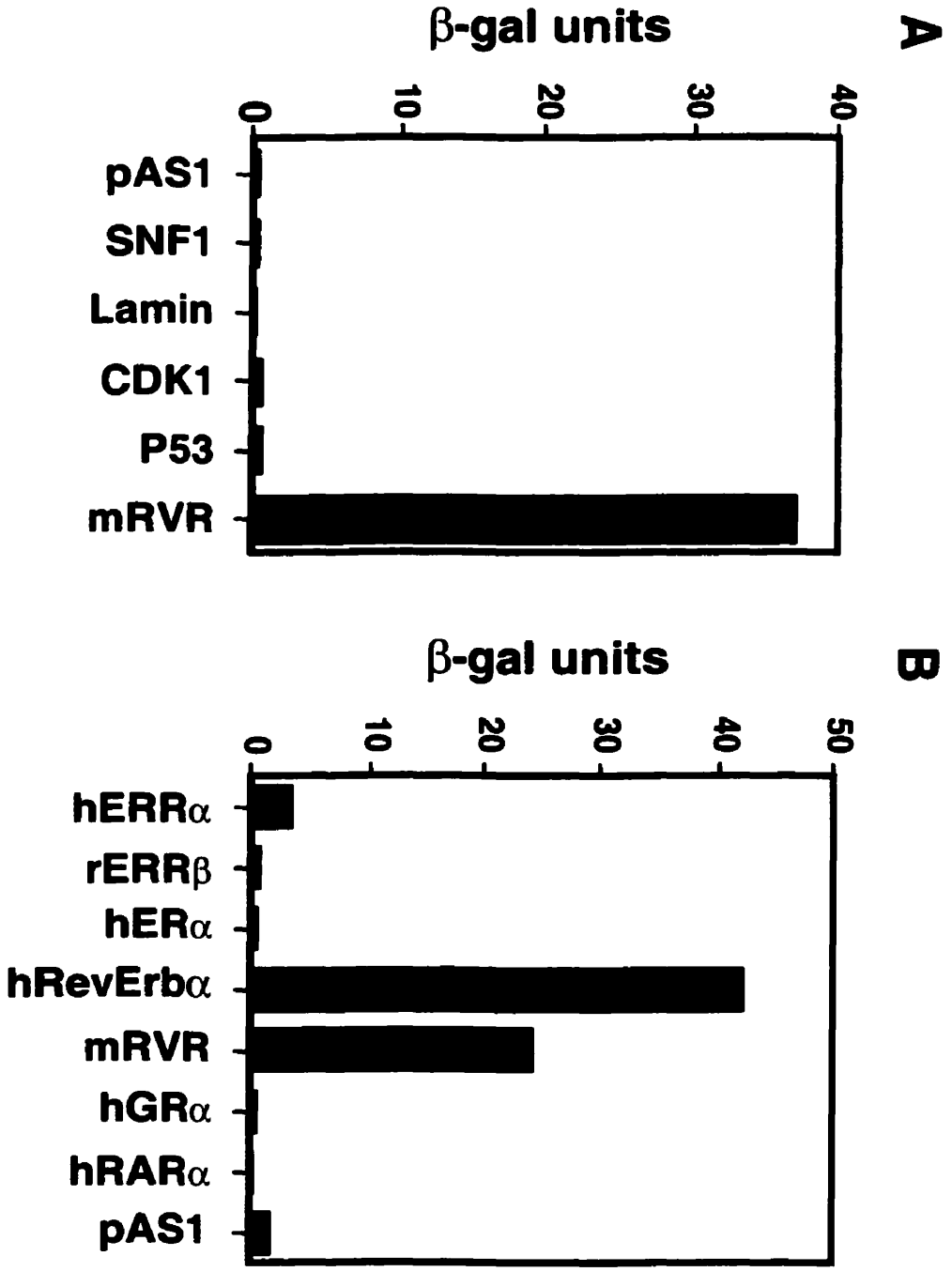
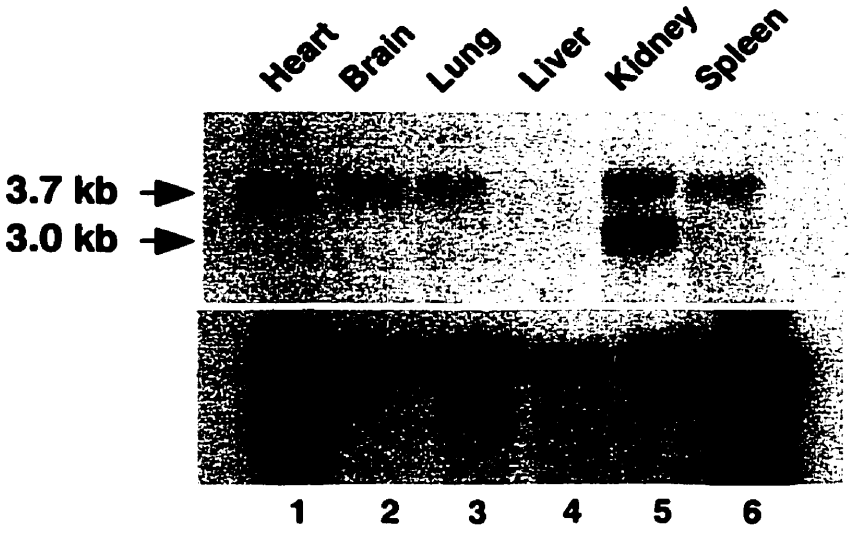

β-gal units
A
0
10
20
30
40
pAS1
SNF1
Lamin
CDK1
P53
mRVR
β-gal units
B
0
10
20
30
40
50
hERRα
rERRβ
hERα
hRevErbα
mRVR
hGRα
hRARα
pAS1

FIG 3. Expression and intracellular localization of CIA. (A, top panel) Northern blot of poly-A^+ mRNA from 17.5 dpc mouse embryos, probed with the human CIA cDNA clone shows expression of a 3.7kb mRNA in heart, brain, lung, kidney and spleen. Note the presence of a second 3 kb mRNA in kidney. (A-bottom panel) A β-actin probe was used as control for mRNA integrity. (B) Phase contrast (i and iii) and fluorescence (ii and iv) pictures of HeLa cells transiently transfected with GFP (i and ii) or CIA-GFP (iii and iv) show that CIA-GFP is strictly nuclear while GFP alone distributes evenly throughout the cell.

A


Heart
Brain
Lung
Liver
Kidney
Spleen
3.7 kb
3.0 kb
1
2
3
4
5
6

B

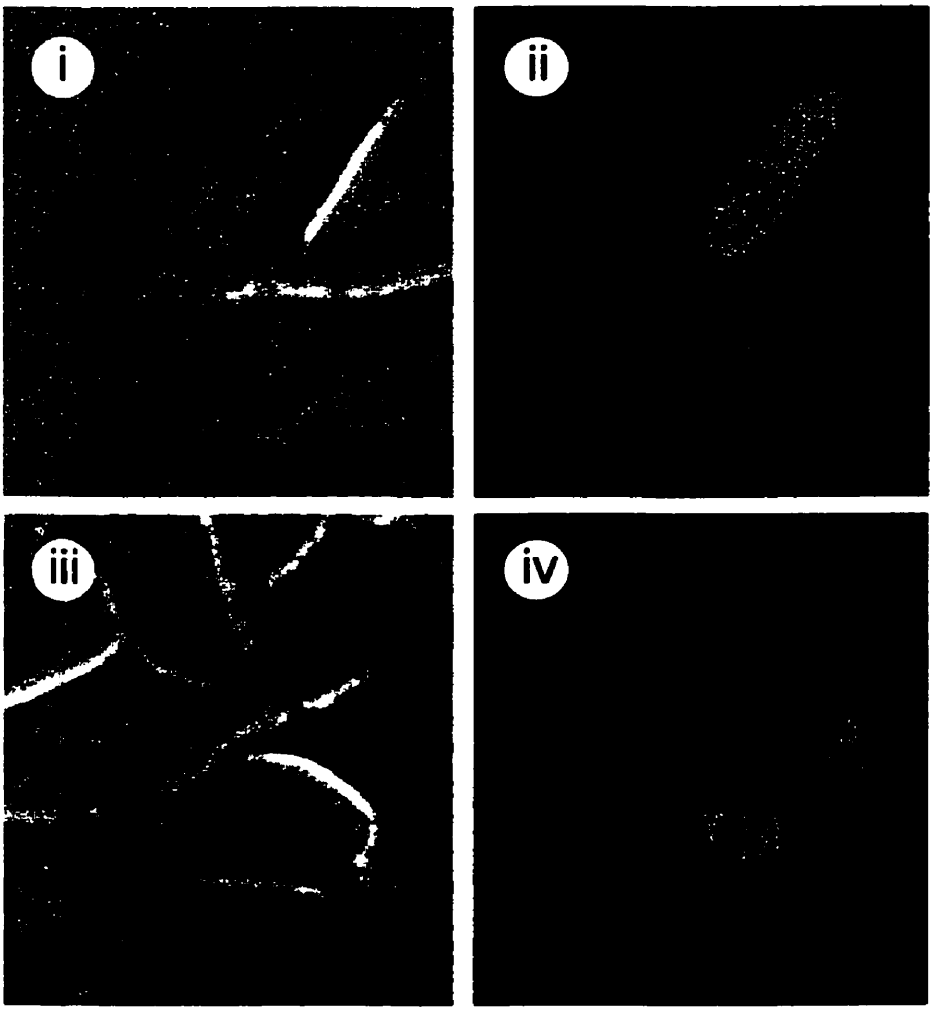
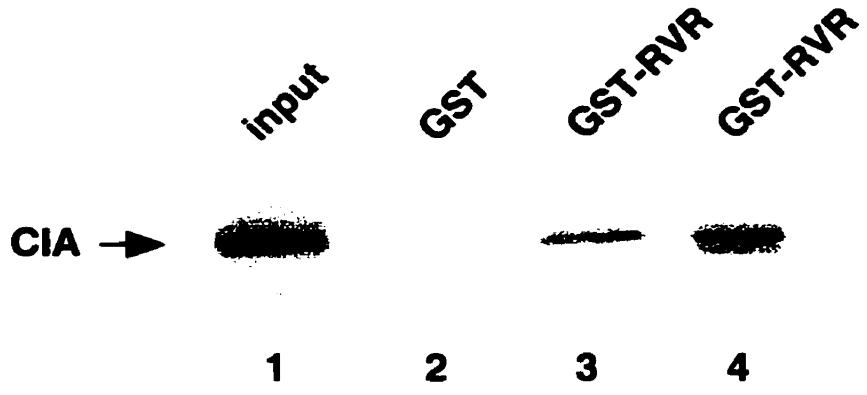

i
ii
iii
iv

FIG 4. ***In vitro*** **interaction of CIA shows specificity for RVR and ERα. (A) Pull-down experiment shows that ^{35}S-labeled CIA interacts *in vitro* with GST-RVR LBD (lane 3) and GST-RVR full length (lane 4) but not with the GST alone control (lane 2). Input represents 10% of the labeled CIA used in the assay (lane 1). (B) Pull-down experiment shows that only ^{35}S-labeled ERα can interact ligand-dependently with GST-CIA. The assay is performed either in presence of 10^{-7}M E_2 (lanes 22 and 24) or carrier (ethanol, lane 23). None of the other tested nuclear receptor including rTRα, mRARα, hVDR, hPR and hGRα demonstrated any interaction with GST-CIA (lanes 1 to 20) either in absence or in presence of their respective hormones (triiodothyronine 10^{-6}M, all-*trans* retinoic acid 10^{-6}M, 1,25-dihydroxyvitamin D 10^{-7}M, progesterone 10^{-7}M and dexamethasone 10^{-7}M).**

A


input
GST
GST-RVR
GST-RVR
CIA
1
2
3
4

B

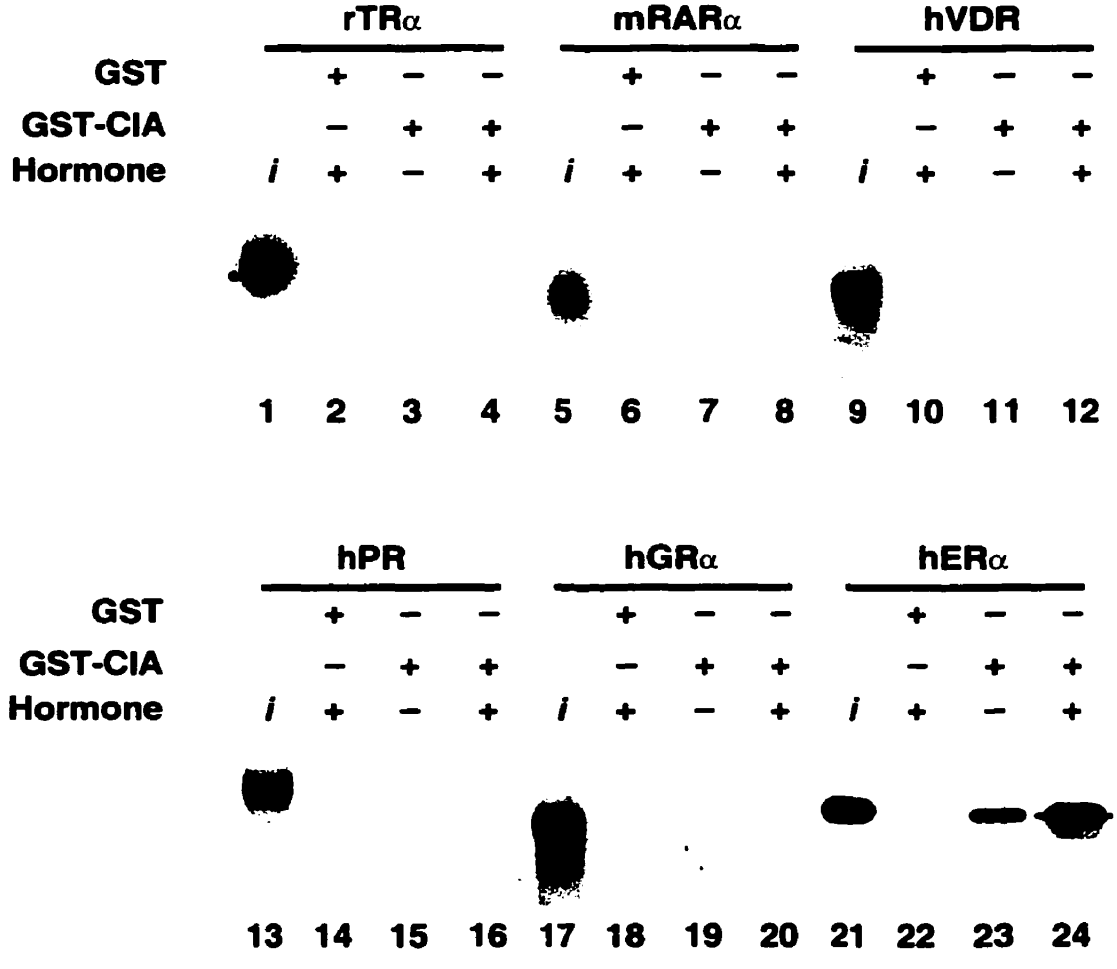

rTRα
mRARα
hVDR
GST
GST-CIA
Hormone
1 2 3 4 5 6 7 8 9 10 11 12
hPR
hGRα
hERα
GST
GST-CIA
Hormone
13 14 15 16 17 18 19 20 21 22 23 24

FIG 5. *In vivo* activation by CIA is specific for ERα and is independent of the promoter context. (A) Transient transfections in COS-1 cells show that CIA can potentiate ERα activation in presence of ligand (10^{-8}M E_2) on a 2C-vERE-TKLuc, but cannot potentiate T_3Rα activation on a 3C-TREpal-TKLuc or PR activation on a MTV-Luc. Results are expressed in fold induction over the reporter alone. (B) Ligand-dependent activation of ERα on a PS2-Luc is enhanced by cotransfection of CIA. No effect of CIA can be seen on the promoter when its ERE site is mutated (PS2-Luc ΔERE).

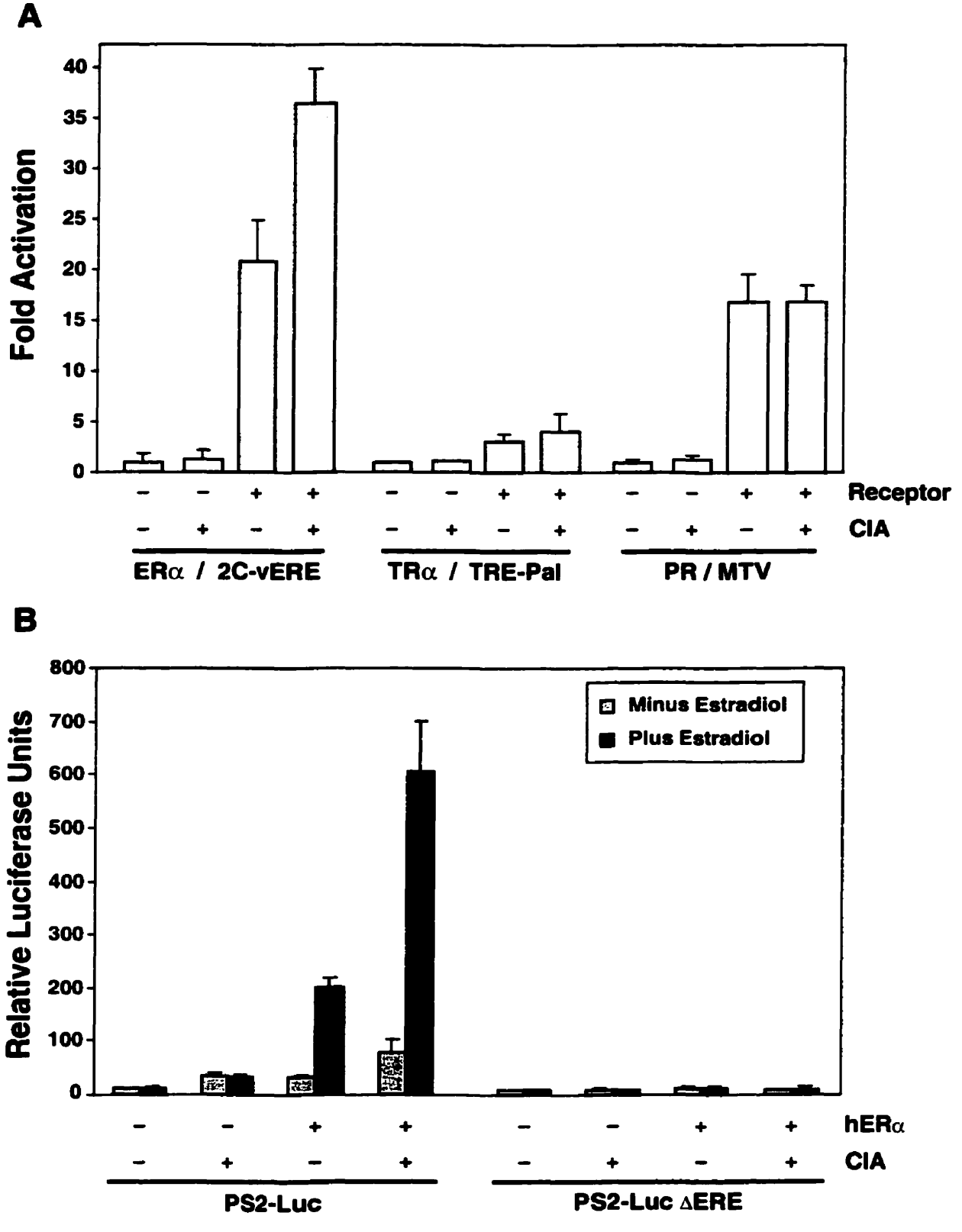
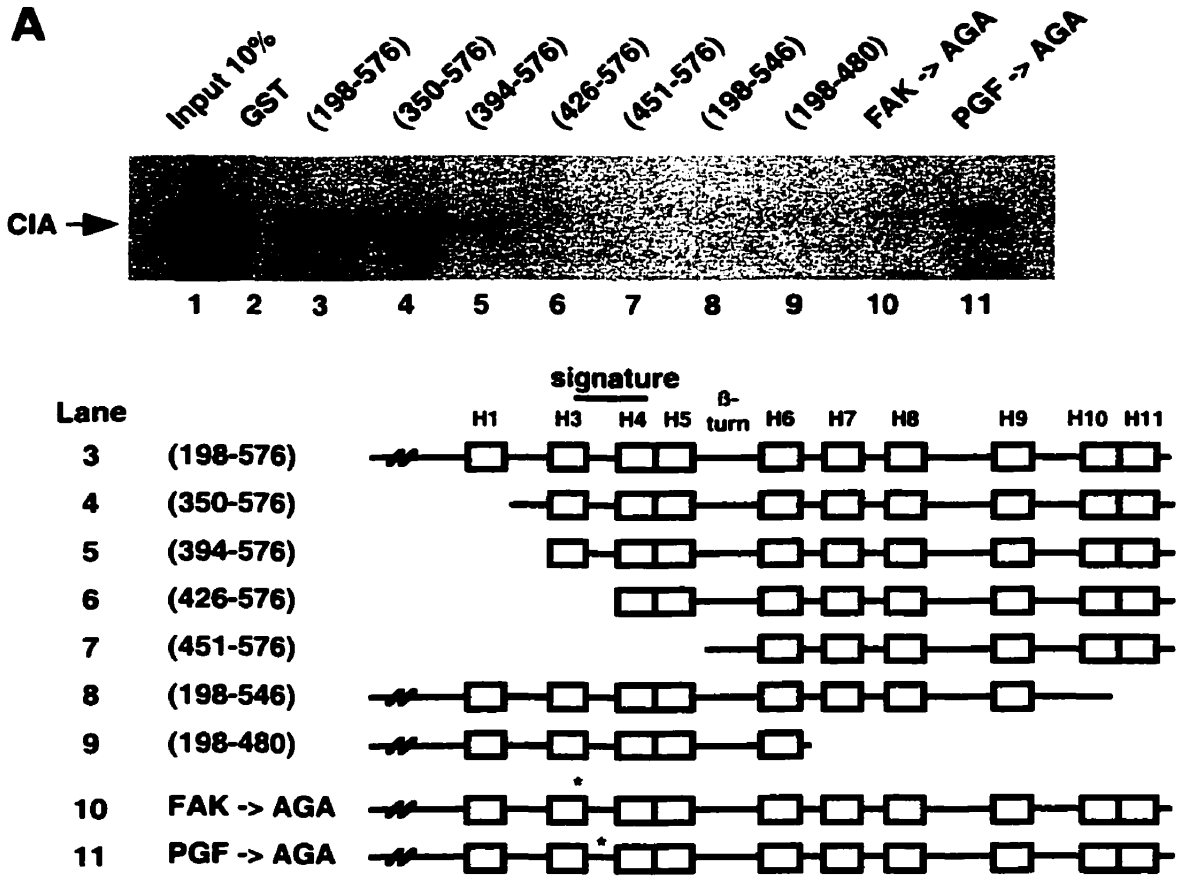
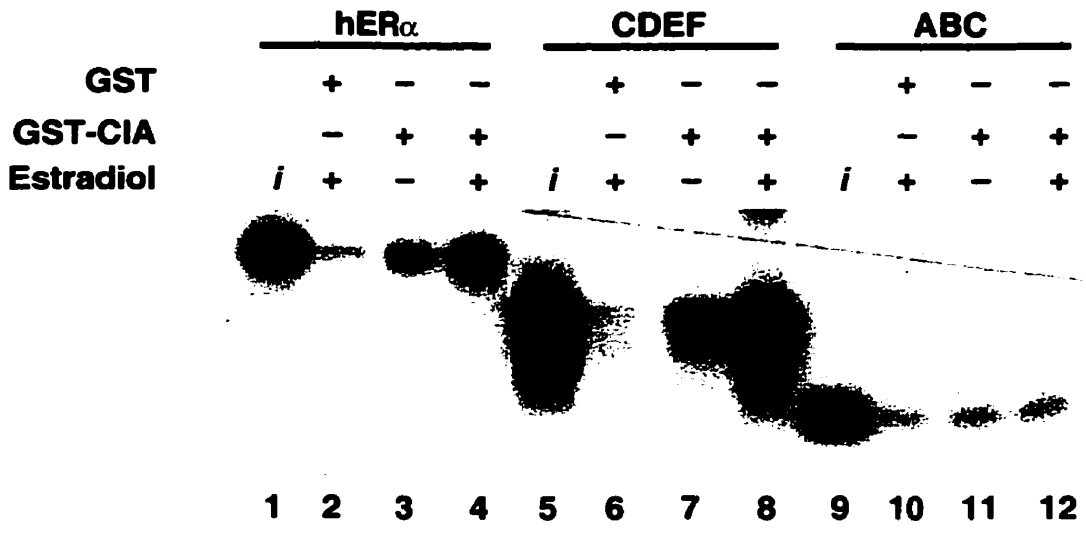

A
Fold Activation
40
35
30
25
20
15
10
5
0
Receptor
CIA
ERα / 2C-vERE
TRα / TRE-Pal
PR / MTV
B
Relative Luciferase Units
800
700
600
500
400
300
200
100
0
Minus Estradiol
Plus Estradiol
hERα
CIA
PS2-Luc
PS2-Luc ΔERE

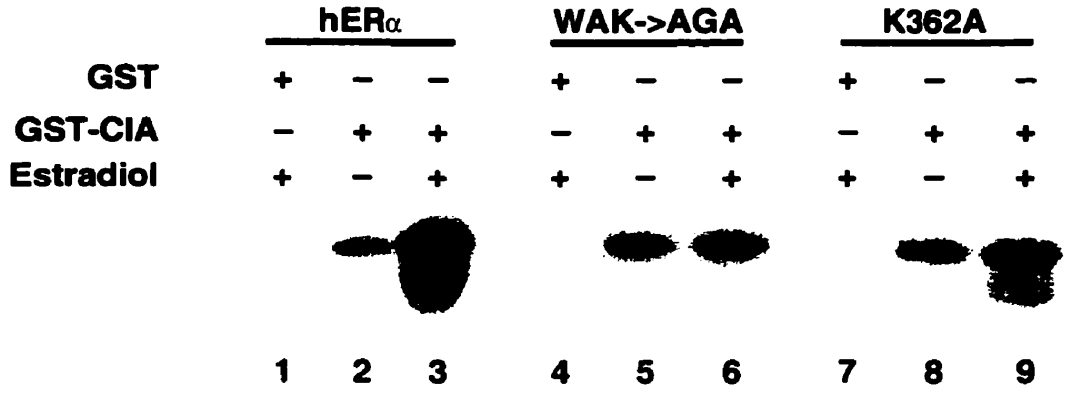
FIG 6. ***In vitro*** **interaction of CIA requires the signature motif of the NR LBD and is differentially modulated by SERMs. (A) Pull-down experiment between ^{35}S-labeled CIA and GST-RVR-LBD truncations shows that the interaction requires regions surrounding helix 3 (compare lanes 5 and 6 to lane 3) and helix 11 (compare line 8 to line 3). CIA interaction is disrupted significantly by mutation of the signature motif: changing residues FAK to AGA (aa415-417) or PGF to AGA (aa420-422) disrupts the CIA-RVR interaction (compare lanes 10 and 11 to lane 3). (B) Pull-down experiments demonstrate that the ligand-dependent interaction occurs between GST-CIA and the C-terminal portion of ^{35}S -labeled ERα (CDEF) (compare lanes 7 and 8 to lanes 3 and 4) and that the N-terminal portion of ERα cannot interact with CIA (lanes 9 to 12). (C) The CIA-ERα interaction can be disrupted by mutating of the signature motif WAK->AGA (aa360 to 362) or only the lysine residue (K362A). (D) Ligand-dependent enhancement of the CIA-ERα interaction (lanes 3 and 4) is inhibited by OHT and raloxifene (compare lanes 5 and 7 with lanes 6 and 8), but is enhanced by the pure antiestrogens ICI182,780 and EM-652 (compare lanes 9 and 11 with lanes 10 and 12).**


A
Input 10%
GST
(198-576)
(350-576)
(394-576)
(426-576)
(451-576)
(198-546)
(198-480)
FAK -> AGA
PGF -> AGA
CIA
1 2 3 4 5 6 7 8 9 10 11
signature
ß-turn
H1 H3 H4 H5 H6 H7 H8 H9 H10 H11
Lane
3 (198-576)
4 (350-576)
5 (394-576)
6 (426-576)
7 (451-576)
8 (198-546)
9 (198-480)
10 FAK -> AGA
11 PGF -> AGA

B


hERα
CDEF
ABC
GST
GST-CIA
Estradiol
i
1 2 3 4 5 6 7 8 9 10 11 12

C


hERα
WAK->AGA
K362A
GST
GST-CIA
Estradiol
1 2 3 4 5 6 7 8 9

D

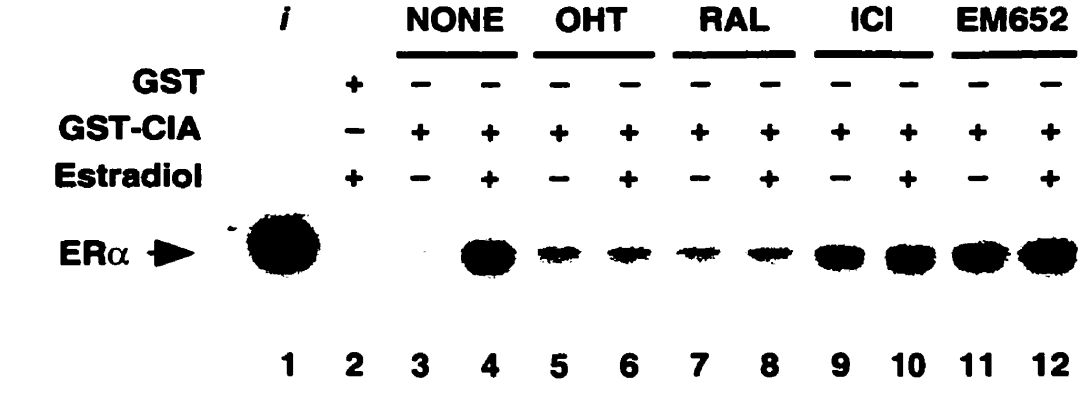
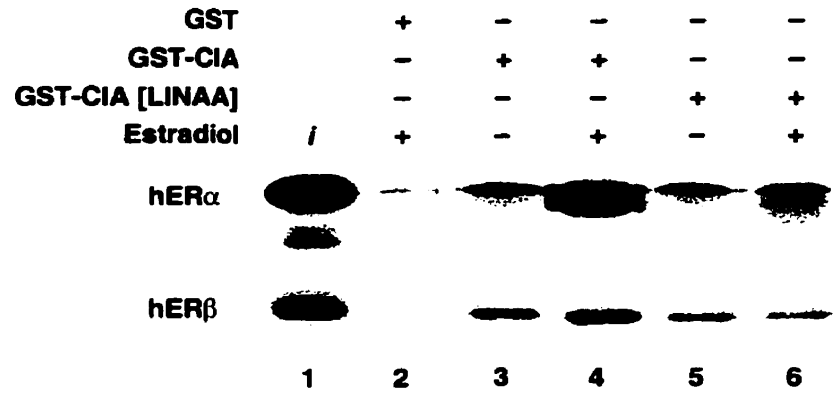

i
NONE
OHT
RAL
ICI
EM652
GST
GST-CIA
Estradiol
ERα
1 2 3 4 5 6 7 8 9 10 11 12

FIG 7. Mutation of the LINLL motif in CIA abolishes the ERα-CIA interaction. (A) Ligand dependent interaction between GST-CIA and ^{35}S-labeled ERα (upper panel, lanes 3 and 4) or ERβ (lower panel, lanes 3 and 4) is disrupted by mutation of the LINLL motif of CIA to LINAA (compare lanes 5 and 6 in each panel). (B) Transient transfection demonstrate that mutation of the LINLL motif reduces the CIA-mediated accentuation of ligand-dependent ERα activity. Transfections were performed in COS-1 cells using the pS2-Luc reporter and pCMX-based expression vectors as described in the materials and methods section. Transfected cells were treated with either 10^{-8} M E_2 or carrier (ethanol) for 24 h post-transfection.

A


GST
GST-CIA
GST-CIA [LINAA]
Estradiol
i
hERα
hERβ
1 2 3 4 5 6

B

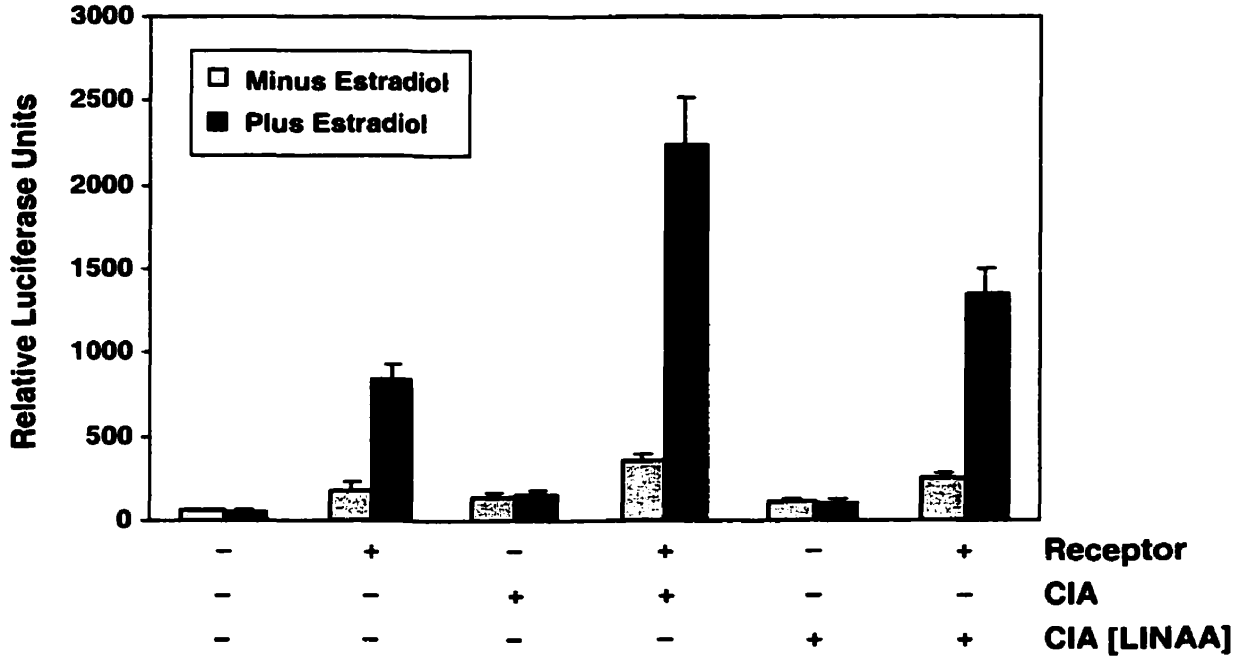
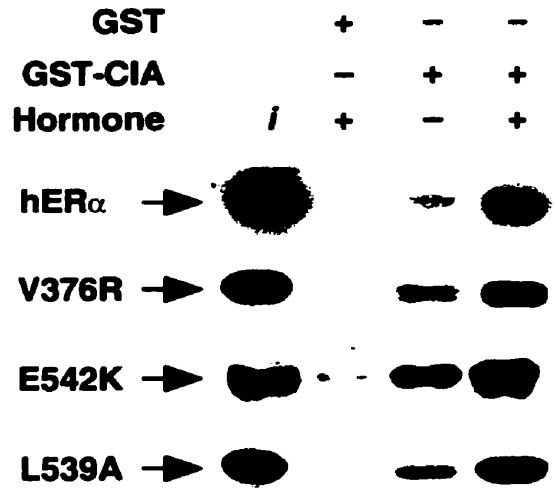
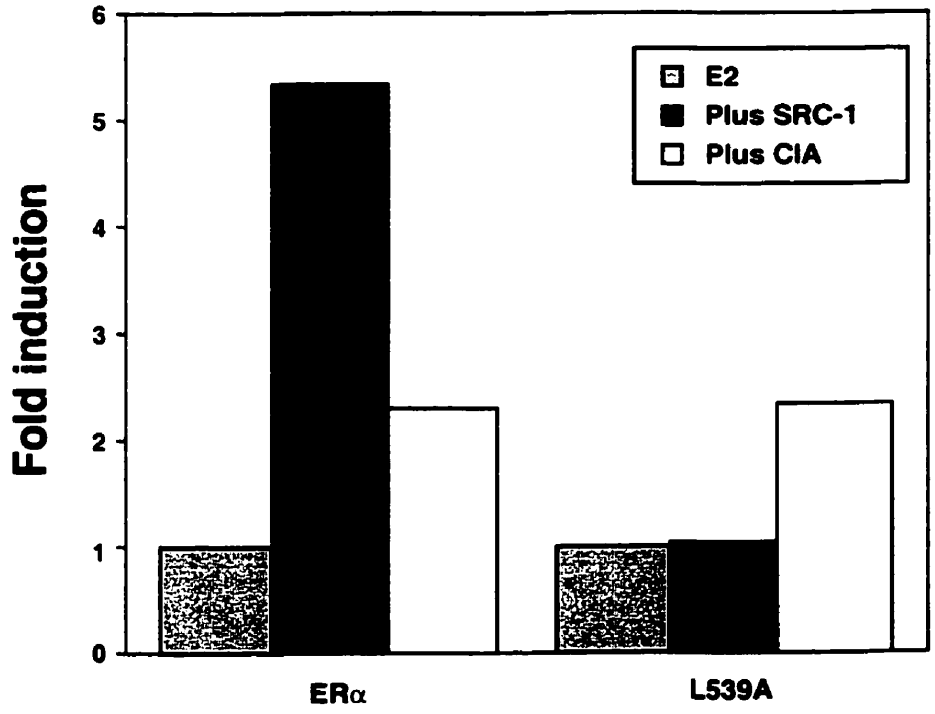

Relative Luciferase Units
3000
2500
2000
1500
1000
500
0
Minus Estradiol
Plus Estradiol
Receptor
CIA
CIA [LINAA]

FIG 8. Ligand dependent interaction between CIA and ERα is not blocked by mutation of residues in the receptor AF-2 domain. (A) Pulldown assays demonstrate that ligand induced interaction between GST-CIA and ERα is not disrupted by mutation of conserved residues in the ERα AF-2 (compare mutants E542K and L539A with the wild-type receptor), or by mutation of a residue located in the co-activator binding cleft (compare mutant V376R with the wild-type receptor). (B) In contrast to SRC-1, CIA can enhance ligand-dependent activation of an AF-2 deficient mutant ERα.

A


GST
GST-CIA
Hormone
i
hERα
V376R
E542K
L539A

B


Fold induction
0
1
2
3
4
5
6
E2
Plus SRC-1
Plus CIA
ERα
L539A

REFERENCES

Bartel, P., Chien, C. T., Sternglanz, R., and Fields, S. (1993). Elimination of false positives that arise in using the two-hybrid system. Biotechniques *14*, 920-924.

Brzozowski, A. M., Pike, A. C. W., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, L., Greene, G. L., Gustafsson, J.-Å., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature *389*, 753-758.

Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., Montminy, M., and Evans, R. M. (1996). Role of CBP/P300 in nuclear receptor signalling. Nature *383*, 99-103.

Chien, C. T., Bartel, P. L., Sternglanz, R., and Fields, S. (1991). The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA *88*, 9578-9582.

Collingwood, T. N., Wagner, R., Matthews, C. H., Clifton-Bligh, R. J., Gurnell, M., Rajanayagam, O., Agostini, M., Fletterick, R. J., Beck-Peccoz, P., Reinhardt, W., Binder, G., Ranke, M. B., Hermus, A., Hesch, R. D., Lazarus, J., Newrick, P., Parfitt, V., Raggatt, P., de Zegher, F., and Chatterjee, V. K. (1998). A role for helix 3 of the TRβ ligand-binding domain in coactivator recruitment identified by characterization of a third cluster of mutations in resistance to thyroid hormone. EMBO J. *17*, 4760-4770.

Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992). Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. EMBO J. *11*, 1025-1033.

Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998). Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev. *12*, 3343-3356.

Ding, X. F., Anderson, C. M., Ma, H., Hong, H., Uht, R. M., Kushner, P. J., and Stallcup, M. R. (1998). Nuclear receptor-binding sites of coactivators glucocorticoid receptor

interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1): multiple motifs with different binding specificities. Mol. Endocrinol. *12*, 302-313.

Dussault, I., and Giguère, V. (1997). Differential regulation of the N-myc proto-oncogene by RORα and RVR, two orphan members of the superfamily of nuclear hormone receptors. Mol. Cell. Biol. *17*, 1860-1867.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240*, 889-895.

Feng, W., Ribeiro, R. C. J., Wagner, R. L., Nguyen, H., Apriletti, J. W., Fletterick, R. J., Baxter, J. D., Kushner, P. J., and West, B. L. (1998). Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science *280*, 1747-1749.

Fraser, R. A., Heard, D. J., Adam, S., Lavigne, A. C., Le Douarin, B., Tora, L., Losson, R., Rochette-Egly, C., and Chambon, P. (1998). The putative cofactor TIF1α is a protein kinase that is hyperphosphorylated upon interaction with liganded nuclear receptors. J. Biol. Chem. *273*, 16199-16204.

Giguère, V. (1999). Orphan nuclear receptors: from gene to function. Endocr. Rev. *20*, 689-725.

Giguère, V., Shago, M., Zirngibl, R., Tate, P., Rossant, J., and Varmuza, S. (1990). Identification of a novel isoform of the retinoic acid receptor γ expressed in the mouse embryo. Mol. Cell. Biol. *10*, 2335-2340.

Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (1997). Nuclear receptor coactivators. Curr. Opin. Cell Biol. *9*, 222-232.

Hammer, G. D., Krylova, I., Zhang, Y., Darimont, B. D., Simpson, K., Weigel, N. L., and Ingraham, H. A. (1999). Phosphorylation of the nuclear receptor SF-1 modulates cofactor recruitment: integration of hormone signaling in reproduction and stress. Mol. Cell *3*, 521-526.

Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature *387*, 733-736.

Hollenberg, S. M., and Evans, R. M. (1988). Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. Cell *55*, 899-906.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.-C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996). A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell *85*, 403-414.

Kliewer, S. A., Lehmann, J. M., and Willson, T. M. (1999). Orphan nuclear receptors: shifting endocrinology into reverse. Science *284*, 757-760.

Lanz, R. B., McKenna, N. J., Onate, S. A., Albrecht, U., Wong, J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1999). A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex. Cell *97*, 17-27.

Lavinsky, R. M., Jepsen, K., Heinzel, T., Torchia, J., Mullen, T. M., Schiff, R., Del-Rio, A. L., Ricote, M., Ngo, S., Gemsch, J., Hilsenbeck, S. G., Osborne, C. K., Glass, C. K., Rosenfeld, M. G., and Rose, D. W. (1998). Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc. Natl. Acad. Sci. USA *95*, 2920-2925.

Le Douarin, B., Zechel, C., Garnier, J.-M., Lutz, Y., Tora, L., Pierrat, B., Heery, D., Gronemeyer, H., Chambon, P., and Losson, R. (1995). The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. EMBO J. *14*, 2020-2033.

Lee, J. W., Ryan, F., Swaffield, J. C., Johnston, S. A., and Moore, D. D. (1995). Interaction of thyroid-hormone receptor with a conserved transcriptional mediator. Nature *374*, 91-94.

Mangelsdorf, D. J., and Evans, R. M. (1995). The RXR heterodimers and orphan receptors. Cell *83*, 841-850.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995). The nuclear receptor superfamily: the second decade. Cell *83*, 835-839.

McInerney, E. M., Rose, D. W., Flynn, S. E., Westin, S., Mullen, T. M., Krones, A., Inostroza, J., Torchia, J., Nolte, R. T., Assa-Munt, N., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1998). Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. Genes Dev. *12*, 3357-3368.

McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999). Nuclear receptor coregulators: cellular and molecular biology. Endocr. Rev. *20*, 321-344.

Meyer, M.-E., Gronemeyer, H., Turcotte, B., Bocquel, M.-T., Tasset, D., and Chambon, P. (1989). Steroid hormone receptors compete for factors that mediate their enhancer function. Cell *57*, 433-442.

Oñate, S. A., Boonyaratanakornkit, V., Spencer, T. E., Tsai, S. Y., Tsai, M. J., Edwards, D. P., and O'Malley, B. W. (1998). The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. J. Biol. Chem. *273*, 12101-12108.

Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M., and Spiegelman, B. M. (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell *92*, 829-839.

Retnakaran, R., Flock, G., and Giguère, V. (1994). Identification of RVR, a novel orphan nuclear receptor that acts as a negative transcriptional regulator. Mol. Endocrinol. *8*, 1234-1244.

Rochette-Egly, C., Adam, S., Rossignol, M., Egly, J.-M., and Chambon, P. (1997). Stimulation of RARα activation function AF-1 through binding to the general transcription factor TFIIH and phosphorylation by CDK7. Cell *90*, 97-107.

Saatcioglu, F., Deng, T., and Karin, M. (1993). A novel cis element mediating ligand-independent activation by c-ErbA: implications for hormonal regulation. Cell *75*, 1095-1105.

Schulman, I. G., Chakravarti, D., Juguilon, H., Romo, A., and Evans, R. M. (1995). Interactions between the retinoid X receptor and a conserved region of the TATA-binding protein mediate hormone-dependent transactivation. Proc. Natl. Acad. Sci. USA *92*, 8288-8292.

Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell *95*, 927-937.

Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, N. J., Onate, S. A., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1997). Steroid receptor coactivator-1 is a histone acetyltransferase. Nature *389*, 194-198.

Tini, M., Otulakowski, G., Breitman, M. L., Tsui, L.-T., and Giguère, V. (1993). An everted repeat mediates retinoic acid induction of the γF-crystallin gene: evidence of a direct role for retinoids in lens development. Genes Dev. *7*, 295-307.

Tremblay, A., Tremblay, G. B., Labrie, C., Labrie, F., and Giguère, V. (1998). EM-800, a novel antiestrogen, acts as a pure antagonist of the transcriptional functions of estrogen receptors α and β. Endocrinology *139*, 111-118.

Tremblay, A., Tremblay, G. B., Labrie, F., and Giguère, V. (1999). Ligand-independent recruitment of SRC-1 by estrogen receptor β through phosphorylation of activation function AF-1. Mol. Cell *3*, 513-519.

Tremblay, G. B., Tremblay, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Labrie, F., and Giguère, V. (1997). Cloning, chromosomal localization and functional analysis of the murine estrogen receptor β. Mol. Endocrinol. *11*, 353-365.

Treuter, E., Johansson, L., Thomsen, J. S., Wärnmark , A., Leers, J., Pelto-Huikko, M., Sjöberg, M., Wright, A. P., Spyrou, G., and Gustafsson, J. (1999). Competition between thyroid hormone receptor-associated protein (TRAP) 220 and transcriptional

intermediary factor (TIF) 2 for binding to nuclear receptors. Implications for the recruitment of TRAP and p160 coactivator complexes. J. Biol. Chem. *274*, 6667-6677.

Umesono, K., Giguère, V., Glass, C. K., Rosenfeld, M. G., and Evans, R. M. (1988). Retinoic acid and thyroid hormone induce gene expression through a common responsive element. Nature *336*, 262-265.

White, R., Sjöberg, M., Kalkhoven, E., and Parker, M. G. (1997). Ligand-independent activation of the estrogen receptor by mutation of a conserved tyrosine. EMBO J. *16*, 1427-1435.

Yang, X.-J., Ogryzko, V. V., Nishikawa, J.-i., Howard, B. H., and Nakatani, Y. (1996). A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature *382*, 319-324.

APPENDIX - ADDITIONAL RESULTS

Appendix A1 - Isolation of a full-length cDNA clone for CIA

Data gathered from CIA the sequence (see figure 1), the Northern blots (see figure 3) and the EST alignments (see section 1 of additional results) did not allow us to conclude that the cDNA clone presented in this thesis is the full-length protein. First, the sequencing of the clone revealed an open reading frame continuing until the end of its 5'-extremity. Secondly, the first putative initiation codon (bold in figure 1) is not at all in a favorable consensus context for a start site (reviewed in Kozak, 1992). Third, analysis of mRNAs transcripts observed on northern blots (see figure 3) and data obtained from EST alignments (data not shown) revealed that there could be 100 to 200 bp missing from the 5'-end of our CIA clone. Taken together, these observations suggested that the CIA cDNA clone lacked 5' sequences that were present in the full length CIA mRNA species. We started an intensive search for a longer clone that could be a complete ORF for the protein.

The initial strategy involved screening of cDNA libraries in order to identify a longer ORF for CIA. Multiple libraries were screened sequentially (human placenta, mouse brain and mouse embryonic kidney) with various fragments of CIA as probes. Standard filter hybridization techniques were followed and multiple hits were obtained in each of these screens. Although many cDNAs were recovered, none of them extended further 5' than the sequence reported in figure 1. The large number of independent clones recovered supported the idea that the screen was appropriately conducted: our failure to recover longer transcripts is likely due to the fact that our clone is complete. Although, the method of construction of the libraries themselves (these libraries were constructed using

poly-A tail tagging rather than random priming or a combination of both), still did not allow us to conclusively rule out the existence of a longer cDNA.

As an alternate means of recovering additional 5' sequence, we performed Rapid Amplification of cDNA Ends (RACE) experiments on mouse kidney and brain mRNA. This technique allowed us to use specific primers close to the 5'-end of the CIA clone to maximize our chances of recovering a longer clone. Unfortunately, three separate attempts to extend the 5'end of the current CIA clone using either total or poly-A^+ RNA failed. All the RACE products terminated within the first 95 pb of CIA clone (figure 1). A striking characteristic of this region is its high content of G and C nucleotides: such GC-rich regions are known to form secondary structures that reverse transcriptase (RT) enzymes have difficulties overcoming (Mytelka and Chamberlin, 1996; Rees et al., 1993). In order to solve this problem, we performed a RACE experiment using the thermostable enzyme *Tth* (Epicentre Technologies) that can be incubated at temperatures up to 72°C for the RT reaction. This technique also failed to obtain a longer 5' cDNA product. While our results suggest htat the CIA clone may be full-length, this stretch of GC-rich nucleotides could be forming a secondary structure too stable to be processed by our current *in vitro* reverse transcription techniques, and therefore prevented us to rule out the existence of a longer cDNA.

Since all these approaches were unsuccessful, we finally decided to conduct a PCR-based screen jointly with Genome Systems Inc., in order to help solve our dilemma concerning the putative existence of a longer ORF for CIA. They screened 10 "long cDNAs" containing libraries and identified multiple positive clones. Subsequent isolation of cDNA from an embryonic brain library yielded clones that were not longer then the CIA cDNA presented in figure 1.

Although no conclusive evidence exists to assert whether the cDNA clone we possess for CIA is full length, after performing all these experiments, we feel increasingly confident that our clone is indeed a complete one.

Appendix A2 - Identification of a putative physiological role for CIA

In an effort to identify functional characteristics and possible homologues for CIA, multiple sequence homology searches were performed into the National Center for Biotechnology Information (NCBI) sequence data bank (Altschul et al., 1997). These searches suggested that CIA is not homologous to any previously identified proteins, especially known coactivators. Even performing more advanced searches, using BLOCKS[b], failed to identify any conserved protein motifs (Henikoff and Henikoff, 1994). Initially, this was seen as a major set-back. It would have been very useful in the initial characterization of CIA had identified homologies or conserved functional domain to proteins with known function. The only significant protein homology (2e-23 on the protein level) is between the central region of CIA (AA240 to 487) and a *Drosophila melanogaster* clone of unknown function (accession number AF160904). As seen on figure 12 (boxed), the LINLL motif required for the CIA-ERα and β ligand-dependent interaction is not conserved between the two clones. Therefore, this *Drosophila melanogaster* clone may not represent a functional homologue of CIA, but further investigation would be necessary to properly address this question.

The sequence homology searches against Expressed Sequence Tagged (EST) databases at the NCBI (Altschul et al., 1997) identified more than 20 EST that are significantly homologous to CIA. All these clones were either from human, rat or mouse cDNA libraries. These homologies confirmed that the mRNA for CIA is indeed expressed in a variety of tissues in mammals. Unfortunately, none of these ESTs extended for the 5'-end of our CIA clone (this point will be discussed in section 4). An assembly of these EST clones - performed using the EST Extractor and the EST Assembly Machine at

---

[b] The BLOCKS protein homology server is available at the Fred Hutchinson Cancer Research Center at http://BLOCKS.FHCRC.ORG/
(BLOCKS is a protein alignment tool that searches for conserved motifs and not conservation of an entire protein fragment)

TigemNet[c] - showed the presence of two different 3'-untranscribed regions (UTR) for CIA. This prediction of two putative mRNA messages confirmed results observed on mouse fetal tissues (see figure 3a). This allowed us to conclude that these two different mRNAs were created by the use of an alternative polyadenylation site in the 3'-UTR of the clone.

It is the sequence homology searches against genomic Sequence Tagged Sites (STS) and against High-Throughput Genome Sequencing (HTGS) databases at the NCBI (Altschul et al., 1997) that yielded the most valuable information about CIA. They revealed homology to a genetic marker used by the Sanger Center[d], which is part of the human genome project, to assemble DNA contigs. This marker (Hs182A2T7) is homologous to CIA from bp 756 to 955 (see figure 13). This perfect match allowed us to trace back CIA localization to the Bacterial Artificial Chromosome (BAC) clone dJ599F21 at the Sanger Center. This BAC mapped to the human chromosome 20q12-13.12 and contains the CIA gene. This localization was corroborated via another proximal marker (WI21844) which is also located on dJ599F21. This second marker was mapped to the same region of chromosome 20q by radiation hybrid screening (The Whitehead Institute/MIT Center for Genome Research)[e]. This mapping information was of great interest for us because this region of human chromosome 20 was previously shown to contain AIB1, another NR coactivator overexpressed in breast and ovarian cancers (Anzick et al., 1997).

Since CIA mapped to human chromosome 20q12-13.12 which spans a region previously shown by numerous reports to be amplified and overexpressed in breast and ovarian cancers (Anzick et al., 1997; Bautista et al., 1998; Guan et al., 1996; Tanner et al., 1994;

---

[c] TigemNet can be accessed at http://gcg.tigem.it/cgi-bin/UNIESTASS.PL

[d] These sequence data were produced by the Human Sequencing Group at the Sanger Center and can be obtained from ftp://ftp.sanger.ac.uk/pub/human/chr20/

[e] The Whitehead Institute/MIT Center for Genome Research can be accessed at: www.genome.wi.mit.edu

Tanner et al., 1996), we wanted to verify whether CIA would also be overexpressed or amplified in some of these cancer cell lines.

We first isolated RNA and genomic DNA from a panel of breast cancer cell lines using Trizol (Gibco Life Technologies), following the manufacturer's instructions. Total RNA was run on a northern gel, transferred to a nylon membrane and then hybridized to a radiolabelled probe prepared using the full-length CIA cDNA clone. The membranes were stripped and restudied using a rediolabelled AIB1 probe: this represents a positive control for our studies as many breast cancer lines have been shown to overexpress this coregulator. As seen in figure 14, top panel, expression of CIA is about 2 to 3 fold higher in all tested cancer cell lines (lanes 3 to 14) then in the control cell lines (lanes 1 and 2). Even if the expression level is higher, the ratio of CIA expression (cancer cells / normal cells) is less than the ratio for AIB1 expression (figure 14, 2nd panel), which is used here as a positive control for overexpression. Although we can never rule out the possibility that CIA could be overexpressed in some cancer cell lines not studied here, the data available forced us to conclude that CIA was not overexpressed in this panel of cancer cell lines. In an effort to identify a potential correlation between CIA and SRC-1 expression patterns, the membrane was also blotted with SRC-1 (figure 14, 3rd panel): no corelation between SRC-1 and CIA expression lecels was observed in these cell lines. Finally, to adjust for loading differences, the membrane was probed with hGAPDH (figure 14, bottom panel).

In addition, we wished to determine whether the chromosome 20 amplification seen in breast cancer cell lines involved the CIA locus. Genomic DNA was digested with restriction enzyme *Eco*RI, run on an agarose gel and transferred to a nylon membrane. This membrane was then sequentially probed for CIA, AIB1, SRC-1 and GAPDH (see figure 15). Again here, comparison of the relative signal levels (cancer cells / control cell)

obtained for CIA with the level obtained for the positive control (AIB1), led us to conclude that CIA gene is not amplified in these breast cancer cell lines.

These results are somewhat surprising since CIA appears to be localized with in a region known to be both amplified and overexpressed in many of the tested cell lines. One possible explanation is that the exact localization of CIA lies just outside the amplified regions of chromosome 20q13. A usefull experiment to address this point would be to map more precisely CIA locus using FISH-mapping. This experiment is ongoing and is so far, without conclusive results (data not shown). Therefore, since both overexpression of the CIA mRNA and amplification of its gene have been ruled-out, the chromosomal localization of CIA will not be addressed further until sequencing of this 20q12-13.12 region is completed by the Sanger Center.

Appendix A3 - Rationale for ERα mutagenesis

The mutants presented in this thesis manuscript were based on previous studies that identified the NR LBD residues mediating the interaction with LXXLL motifs on coactivators (Darimont et al., 1998; Heery et al., 1997; McInerney et al., 1998; Nolte et al., 1998; Westin et al., 1998; Feng at al., 1998). The ERα mutants studied in these papers were modelled using the crystal structure of the ERα LBD: those mutants which appeared to be best positioned to characterize the CIA mode of action were analysed in the present work.

Analysis of crystal structure data showing the position of AF-2 in liganded ERα allowed us to propose that CIA binds to a different surface on the LBD than the p160 coactivators. The modeling of the ERα LBD presented here was made with Swiss PDB Viewer (Guex and Peitsch, 1997), using the PDB coordinate file 1ERE.PDB[f] (Brzozowski et al., 1997). Residues important for the binding of LXXLL-containing coactivators to the hydrophobic cleft are all located around the edges and at the bottom of the cleft (figure 16) these include residues contained in the signature motif as well as residues located in helix 12. The importance of these residues in CIA binding was studied by site-directed mutagenesis. The mutation that disrupts CIA interaction (K362A) (shown in red in figure 16) is located to the right of the cleft, on the side directly opposed to the helix 12. Conversely, the residues on the helix 12 (L539A and E542K)(shown in green) are not necessary for ligand-dependent CIA binding and do not disrupt the CIA-ERα interaction observed in GST pulldown or transient transfection studies. A fourth mutant (V376R) is also dispensable for the interaction. In contrast, SRC-1 interaction with ERa is disrupted by mutation of L539, E542 or V376. This visual representation of the LBD and especially of the hydrophobic cleft with the various mutants, clearly shows that CIA is binding to a

[f] The PDB coordinates for ERα LBD crystal structure (1ERE.PDB) are available from the NCBI database at www.ncbi.nlm.nih.gov

different site on the LBD than other coactivators. This observation is consistent with the cloning strategy used to isolate CIA: which identified proteins interacting with RVR, an orphan NR without an helix 12.

The binding of CIA to a different part of the hydrophobic cleft could also partially be due to its specific LINLL motif (see figure 1). Although little is known about the role of residues surrounding the coactivators LXXLL motifs, it could be envisaged that they affect binding specificity of the coactivator. This hypothesis is supported by experimental observation showing that coactivator-NR interaction could be disrupted by mutation of either sequences surrounding the coactivator LXXLL motif or residues on the surface of nuclear receptors (Darimont et al., 1998). If such is the case, CIA specificity for ERα and β could be due to specific residues located near or signature motif of these receptors. For example, the V376, L536 and the Y537 residues of ERα are not conserved between receptors. As long as we do not have precise structural information (such as cocrystal coordinates) on the positioning of CIA LINLL motif on the surface of ERα, this assumption about specificity remains highly speculative.

The observation that ERα-CIA interaction is enhanced by "pure-antiestrogens" such as ICI 164,384 and EM800 but blocked by tamoxifen and raloxifene suggests that this interaction is susceptible to the LBD conformation. Although crystal structure data shows AF-2 repositioning upon ligand-binding, there are also other more subtle conformation changes that are likely to occur throughout the LBD. Such a subtle conformational change in the ERα LBD must regulate its interaction with CIA. The fact that the AF-2 is not required for this specific interaction implies that the LBD of ERα possesses more regulatory interfaces then the one formed by the classical AF-2-LXXLL motif. These findings suggest that CIA may be an excellent candidate to contribute to the distinct physiological actions of SERMs.

CONCLUSION

In this thesis, we have characterized CIA, a novel coactivator for the superfamily of nuclear receptors. We have established its specificity for some orphan receptors as well as for ER α and β, and have demonstrated that its interaction with ER is promoted by ligand-binding and "pure" antiestrogens, and is independent of AF-2 integrity. We have also provided a structural basis for this unusual property, showing that the region where CIA binds to the LBD is different than the one used by other LXXLL-containing coactivators such as the SRC family. This novel protein is exciting because it demonstrates the existence of an alternate regulatory surface located on the LBD of nuclear receptors. Further characterization of this newly identified interaction surface and elucidation of its role in the regulation of ER-mediated transcription could be important in understanding some of the effects exhibited by SERMs on ERα and β.

In order to properly characterize CIA functions, there are still many questions remaining to be answered. First, it is important to establish whether the current cDNA clone encodes a full-length ORF for the protein. As discussed previously, multiple approaches have failed to produceidentifyon available from the Sanger Center to identify exons upstream of the current 5'-end. Online genome sequences databases currently contain 30 kb of sequenced and aligned genomic DNA upstream of the current ORF. Usage of four different intron/exon prediction packages (such as GENEMARK[g], GRAIL[h], FGENESH[i], GenLang[j]) produced four different possible exonic structure for this 30kb of genomic DNA (data not shown). Therefore no obvious exon / intron structure can be identified from this genomic sequence using computer algorithms. Another possible approach to identify putative CIA exons, would be to clone fragments of this genomic DNA into a

---

[g] Genemark can be accessed on the www at http://www2.ebi.ac.uk/genemark/
[h] Grail can be accessed on the www at http://avalon.epm.ornl.gov/grail-bin
[i] FGENESH can be accessed on the www at http://dot.imgen.bcm.tmc.edu:9331/seq-search/
[j] GenLang can be accessed on the www at http://cbil.humgen.upenn.edu/~sdong/genlang.html

gene trapper system such as Exon Trapper (Gibco BRL). These vectors, upon transfection into mammalian cells, allow the normal cellular machinery to splice out exons from fragments of genomic DNA. This could be one method to overcome the difficulties associated with the 5'end of CIA. If I was to continue studying this protein, it is the approach I would now use, because the analysis of CIA genomic sequences is likely to establish definitively whether or not our clone is complete.

A second unanswered question concerns the molecular mechanism by which CIA exerts its function. Although identifying a function remains speculative until a complete ORF is obtained (as an important domain might be absent from a partial clone), some available information suggests a role for CIA. Apart from the transcriptional coactivator activity discussed in the manuscript, there are some functional characteristics of CIA that were not addressed. First, when a part of CIA is fused to a Gal4-DBD (AA 55-365), it repress transcription (data not shown). This observation is similar to what is obtained when some other coactivators (such as TIF-1) are artificially brought close to DNA. The interpretation we gave to this phenomenon was that bringing the hydrophobic RD-rich region (see figure 1) close to DNA would induce non-specific binding of this intensively charged cluster with the negatively charged DNA. Although we performed non-specific *in vitro* DNA-binding assays with bacterially produced GST-CIA, no such activity was ever observed (data not shown). It is entirely possible that our assays were improperly designed to observe CIA binding to DNA, either because CIA was not properly modified in a bacterial system, or because *in vitro* DNA is structurally significantly different from *in vivo* DNA. If such a CIA-DNA interaction was taking place *in vivo*, it could suggest that CIA plays a role in chromatin rearrangement. To properly address this point, one should study CIA effect on transcription of integrated reporter constructs or on a chromatin-dependent *in vitro* transcription template and should also test for HAT activity. The later test was not performed because chromatin remodeling coactivators

possess a conserved histone-acetyl transferase domain (Yang, 1998) which is not present in CIA. Therefore, the role of CIA might be to recruit other coactivators possessing intrinsic chromatin remodeling enzymatic activities. A recent report suggested that the chromatin-remodeling complex SWI/SNF could be targeted to promoters via the activation domains of activators such as VP16 and GCN4, subsequently stimulating the transcription from nucleosomes arrays (Neely et al., 1999). Perhaps CIA could play a role in the recruitment of such complexes in mammalian cells.

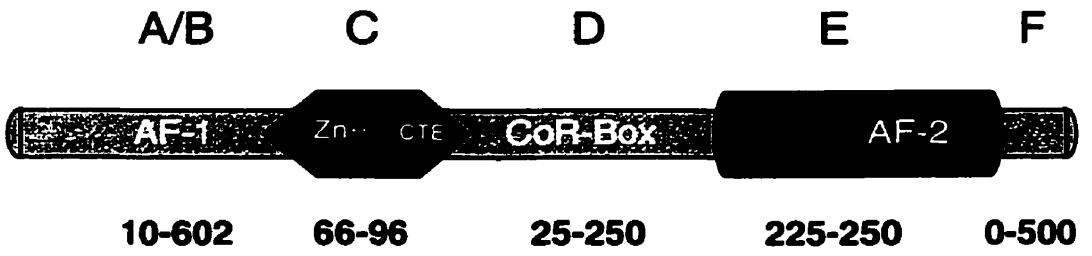
This hypothesis raises a third unanswered question: whether CIA exerts its actions alone or with putative partners. The current picture of coactivators action is that they usually exist as multiprotein complexes, perhaps even complexed with some RNA (Lanz et al., 1999). In such a context, it is reasonable to hypothesize that CIA forms part of such a multiprotein complex *in vivo*. From my point of view, the best current approach to identify and purify such multiprotein complexes is to use a size-fractionation column, which allows the isolation of complexes as a whole, based on their size. O'Malley and colleagues used this technique to isolate SRC-1 containing complexes and identified other copurifying coactivators (McKenna et al., 1998). Usage of such a copurification approach, although technically challenging to establish, should allow the identification of *in vivo* partners for CIA. The identity of these putative partners may provide insights on CIA mechanism of transcriptional activation. If the role of CIA is indeed to mediate the formation of such a complex, it is plausible that some of these copurifying proteins will turn out to possess intrinsic chromatin remodeling activity (such as HAT). This could be a good explanation why CIA does not possess itself such catalytic histone-remodeling capacities.

The final point that should be closely looked at is the CIA-ERα interface. Combined with the observation that "pure antiestrogens" such as EM800 and ICI 164,384 enhance the

CIA-ERα interaction, the identification of ERα K362A as a mutant that prevents CIA binding and conversely, the identification of ERα V376R, L539A and E542K as mutants that can still bind CIA, are good indicators that this interaction occurs through a different interface than the SRC family of coactivators. The structural rationale for these findings could prove to be even more intriguing. To explain the unusual ER-binding characteristics that CIA exhibits, one must consider that the ligand-binding on ERα has to induce additional structural changes on the LBD than the repositioning of the helix 12. Such changes were always considered to be minor for coactivator binding which usually appeared to be regulated by the large conformational change in helix 12 position that results from ligand binding. In the case of CIA, such helix 12 requirement is absent and its interaction has to be "sensing" more subtle changes in the conformation of the other helices. To properly study this hypothesis, we would need to perform more mutagenesis on the hydrophobic cleft side, on residues in the neighbourhood of K362. We would also need to look at the affinity differences between CIA and the various mutants of ERα, to establish which mutations are critical to "sense" the ligand-induced conformational changes.

There is still a considerable amount of work that needs to be done before a clear picture of how CIA exerts its action in the complex process of transcriptional activation. I hope the work presented in this thesis will be useful to get a better understanding of this puzzling action. I also hope that it will raise interest in CIA and that other researchers will want to pursue the investigation of its functions, especially as a modulator of ER activity. The intriguing AF-2 independent activity exhibited by CIA, combined with its putative SERM selectivity should certainly receive consideration because it contrasts the current model for previously described ligand-dependent coactivators of ER, all of which act through the helix 12.

Figure 9 General structure of nuclear receptors. Nuclear receptor structure consists of five functionally conserved domains: (A/B) the modulator domain, which contains the activation function-1. It gets phosphorylated on serine residues downstream of growth factor action and binds some coactivators. (C) The DNA-binding domain which contacts DNA and plays a role in dimerization. (D) The hinge region which allows structural flexibility and contains a region for corepressors binding. (E) The ligand-binding domain where the ligand binds, activating the transcription activation function-2 and promoting coactivators binding. This region also plays a role in dimerization and nuclear localization of the receptor. (F) The LBD extension which is not always present and does not have a known function.



A/B : "Modulator"

- Hypervariable
- Transactivation function 1 (AF-1)
- Modulates target gene specificity
- Phosphorylated on serine residues
- Influences DNA-binding activity

C : "DNA binding domain"

- Highly conserved
- DNA binding through zinc-fingers
- Co-factor interaction
- Dimerization

D : "Hinge"

- Hypervariable
- Co-regulator interaction
- Influence DNA-bending activity

E : "Ligand binding domain"

- Moderately conserved
- Ligand binding
- Dimerization
- Transactivation function 2 (AF-2) within helix 12
- Nuclear localization
- Hsp90
- Co-regulator interaction

F : "F region"

- No identified function

Figure 10 Pathways modulating ERα activity. The ERα activity is mainly regulated via ligand binding to the LBD. This ligand binding activates the AF-2, via induction of conformational changes, and promotes the recruitment of most coactivators (especially the SRCs). The AF-2 activation can be completely blocked (red lines) by the "pure antagonists" EM800 and ICI 164,384 or partially blocked (blue dotted line) by partial antagonists such as OHT, which can also activate the AF-2 in some cellular contexts (blue arrow). ERα can also be activated through serine-phosphorylation of AF-1, downstream of growth factors action. This activation occurs independently of the ligand but can also promote the recruitment of coactivators to the AF-1. This activation of the AF-1 can also be blocked by pure antagonists (red bars).

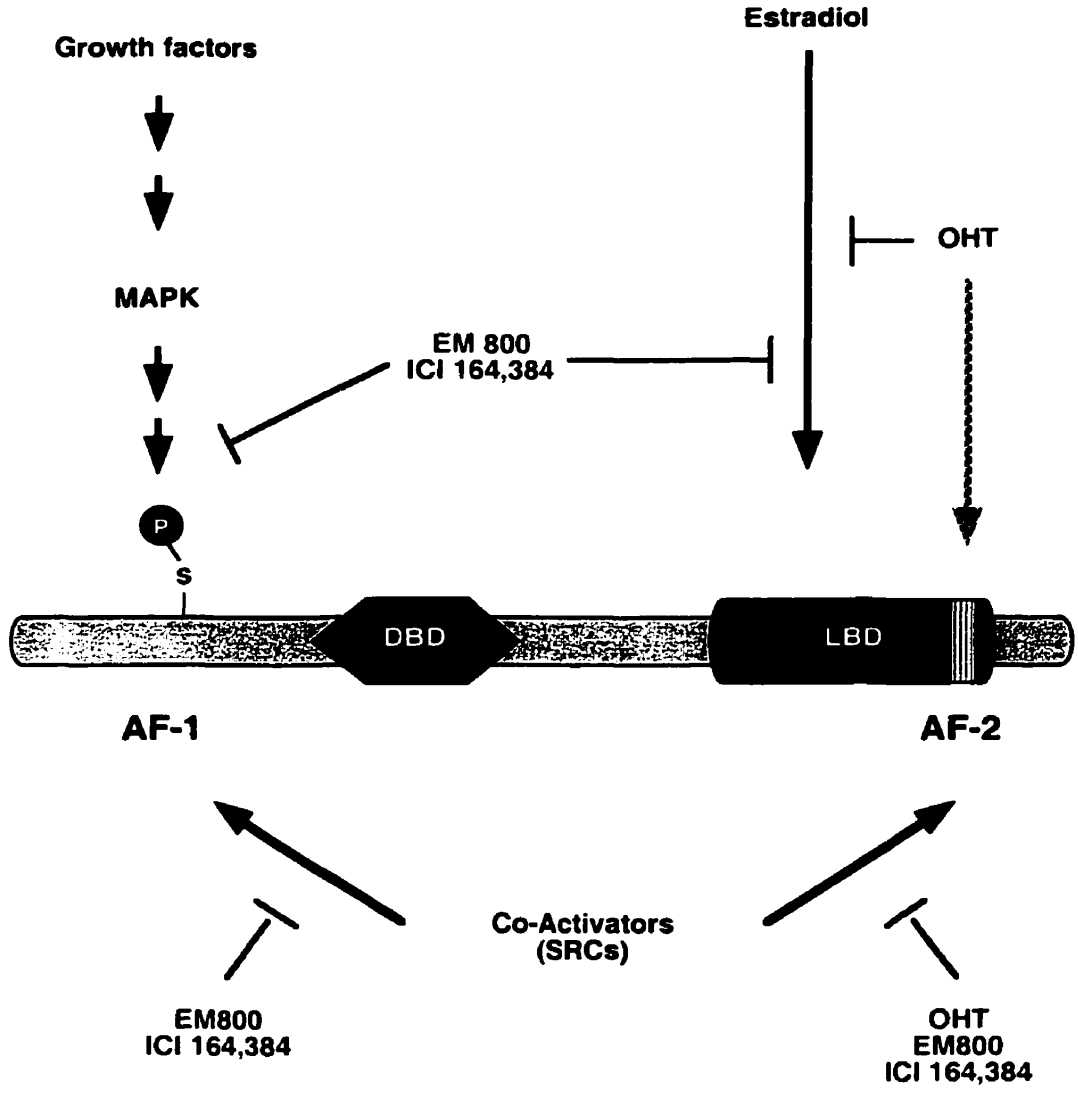

Growth factors
Estradiol
OHT
MAPK
EM 800
ICI 164,384
P
S
DBD
LBD
AF-1
AF-2
Co-Activators
(SRCs)
EM800
ICI 164,384
OHT
EM800
ICI 164,384

Figure 11 Model of ER activation via a multiprotein complex of coactivators. In absence of its cognate ligand, the NR are thought to be contacted by both corepressors and coactivators protein complexes, probably in equilibrium with each other, resulting in a low level of transcription termed basal level. In the case of the estrogen receptor, the activation can occur both ligand-independently (via phosphorylation of the AF-1 and recruitment of SRC-1 containing complex of coactivators) and ligand-dependently (via ligand-binding and recruitment of coactivators complexes to both the AF-1 and AF-2). In both of these activation scenarios, the coactivator multiprotein complexes are now actively recruited resulting in ligand dependent or ligand independent active transcription. Note that the positioning of the various coactivators / corepressors in the multiprotein complexes are strictly arbitrary and are not implying any interaction preferences within the complexes.

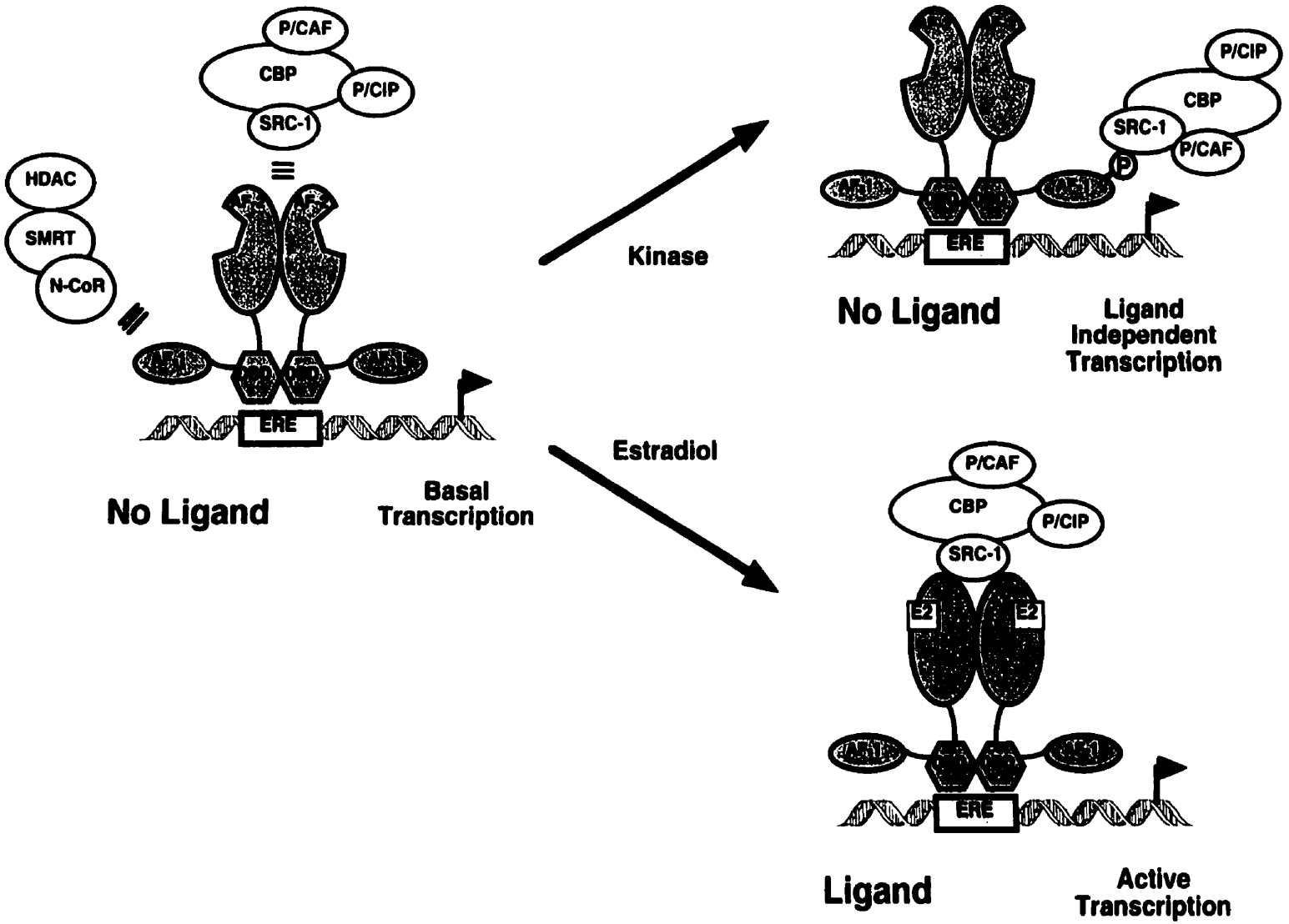

P/CAF
CBP
P/CIP
SRC-1
HDAC
SMRT
N-CoR
ERE
No Ligand
Basal
Transcription
Kinase
Estradiol
P/CIP
CBP
SRC-1
P/CAF
P
ERE
No Ligand
Ligand
Independent
Transcription
P/CAF
CBP
P/CIP
SRC-1
E2
E2
ERE
Ligand
Active
Transcription

Figure 12 CIA is homologous to a *Drosophila melanogaster* clone of unknown function. The central region of CIA (AA 240 to 487) is highly homologous (2 e-23) to a Drosophila clone. Although this homology is significant, the LINLL motif (boxed) which is responsible for the CIA-ERα ligand-dependent interaction is not conserved. Therefore, this Drosophila clone might not represent a functional homologue of CIA.

gb|AF160904.1|AF160904 Drosophila melanogaster clone HL05936 BcDNA.HL05936 (BcDNA.HL05936) mRNA, complete cds
Length = 1253

Score = 113 bits (279), Expect = 2e-23

Identities = 76/247 (30%), Positives = 118/247 (47%), Gaps = 1/247 (0%)

Frame = +2

```
 CIA : 241 DCSVIVVNKQTKDYAESVGRKVRDLGMVVDLIFLNTEVSLSQALEDVSRGGSPFAIVITQ 300
           DC +IV N++    YAE +  ++++  + VD++F N +V L + L ++S  G  +A+++T
Sbjct: 425 DCEIIVQNRENTKYAEYIEERLKNSSLRVDVLFPNEDVLLGKVLANISSRGCLYAVLVTP 604

 CIA : 301 QHQIHRSCTVNIMFGTPQEHRNMPQADAMVLVARNYERYKNECREKEREEIARQAAKMAD 360
           QH+ H S TVNI++G P EHRNMP  DA+ L++ ++   K             R A  +
Sbjct: 605 QHEEHNSITVNILYGVPAEHRNMPLEDAITLISTDFRLKKQ-----------RDAVVLPP 751

 CIA : 361 EAILQERERGGPEEGVRGGHPPAIQSLINLLADNRYLTAEETDKIINYLR-ERKERLMRS 419
              + + +R           HP  +Q L+  LADN  LTA + + I+ YL  ER+E+L R
Sbjct: 752 STSIHKGQR---------RHPQEMQGLLERLADNHPLTASQYEVILKYLEGEREEQLKRE 904

 CIA : 420 STDSLPGPISRQPLGATSGASLKTQPSSQPLQSGQVLPSATPTPSAPPTSQQELQAKILS 479
             +             A + A LK                          P  + ELQ KILS
Sbjct: 905 VGE------------ANALAKLKA----------------------PDPEIELQKKILS 979

 CIA : 480 LFNSGTVT 487
           + N   VT
Sbjct: 980 IMNKPAVT 1003
```

Figure 13 Human CIA gene is located on chromosome 20q12-13.12. Performing sequences alignments against STS databases at the NCBI revealed that CIA is homologous to Hs182A2T7, a marker used by the Sanger Center to localize and map human chromosomes. This marker is contained within dJ599F21, a BAC clone which has been mapped to human chromosome 20q12-13.12. This localization has been corroborated by another marker WI21844 which also maps to the same interval. Therefore, the human gene for CIA is located at 20q12-13.12.

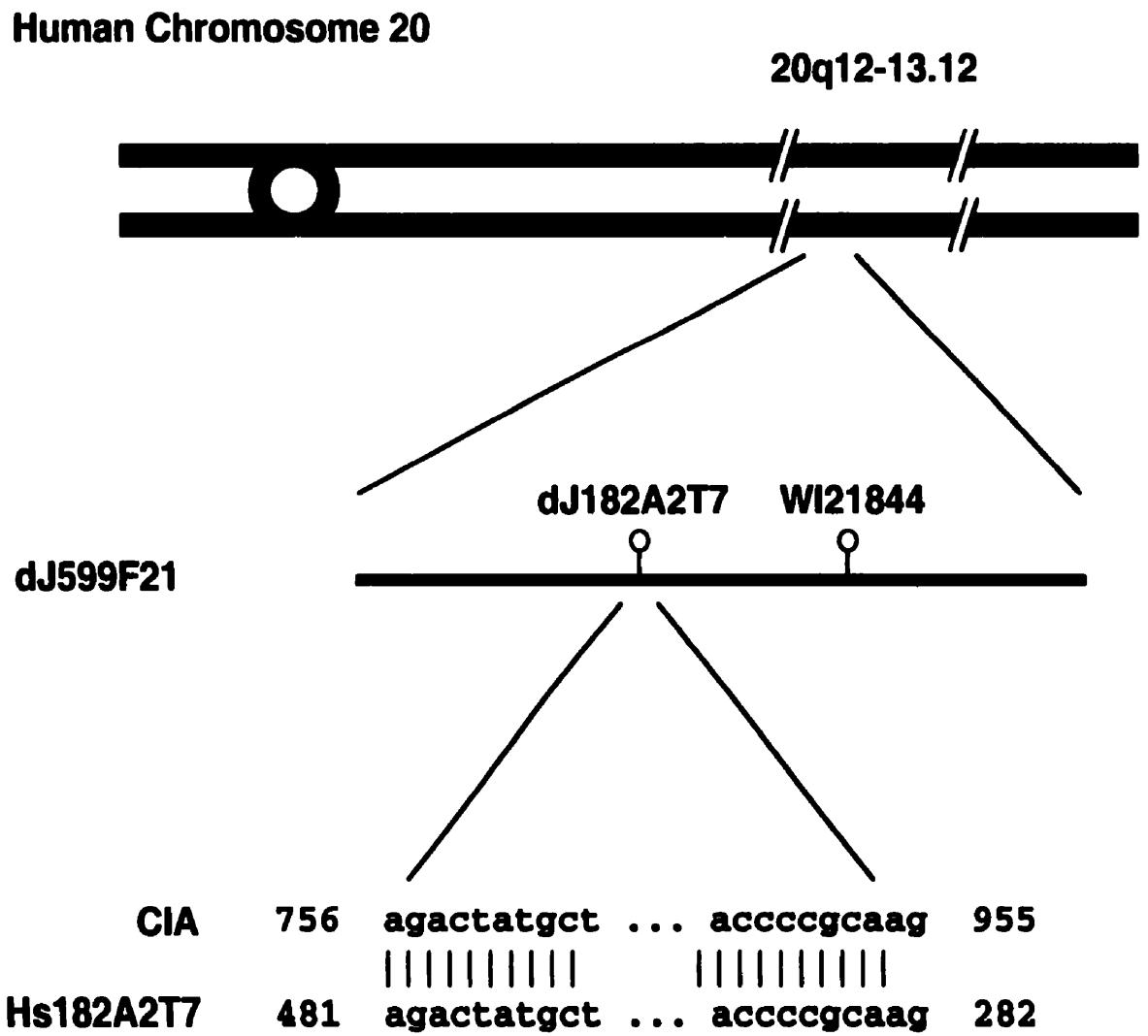

Human Chromosome 20
20q12-13.12
dJ182A2T7
WI21844
dJ599F21
CIA 756 agactatgct ... accccgcaag 955
Hs182A2T7 481 agactatgct ... accccgcaag 282

Figure 14 CIA is expressed to a higher level in breast cancer cells than in normal cells but is not overexpressed to a level comparable with AIB1 overexpression. 10μg of RNA extracted from a panel of breast cancer cell line was run on a formaldehyde/agarose gel and transferred to a nylon membrane. This membrane was probed with ^{32}P-labeled specific cDNA fragments from CIA (top panel), AIB1 (2nd panel), SRC-1 (3rd panel) and GAPDH (bottom panel, control for loading and RNA integrity). CIA is expressed at a higher level in cancer cell lines (lanes 3 to 14) than in control cell lines (lanes 1-2). However, the ratio of expression (cancer cells / normal cells) is not comparable to what is obtained with AIB1, a positive control for overexpression (compare panels 1 and 2). There is also no correlation between the level of CIA and SRC-1 in those cell lines (compare panels 1 and 3).

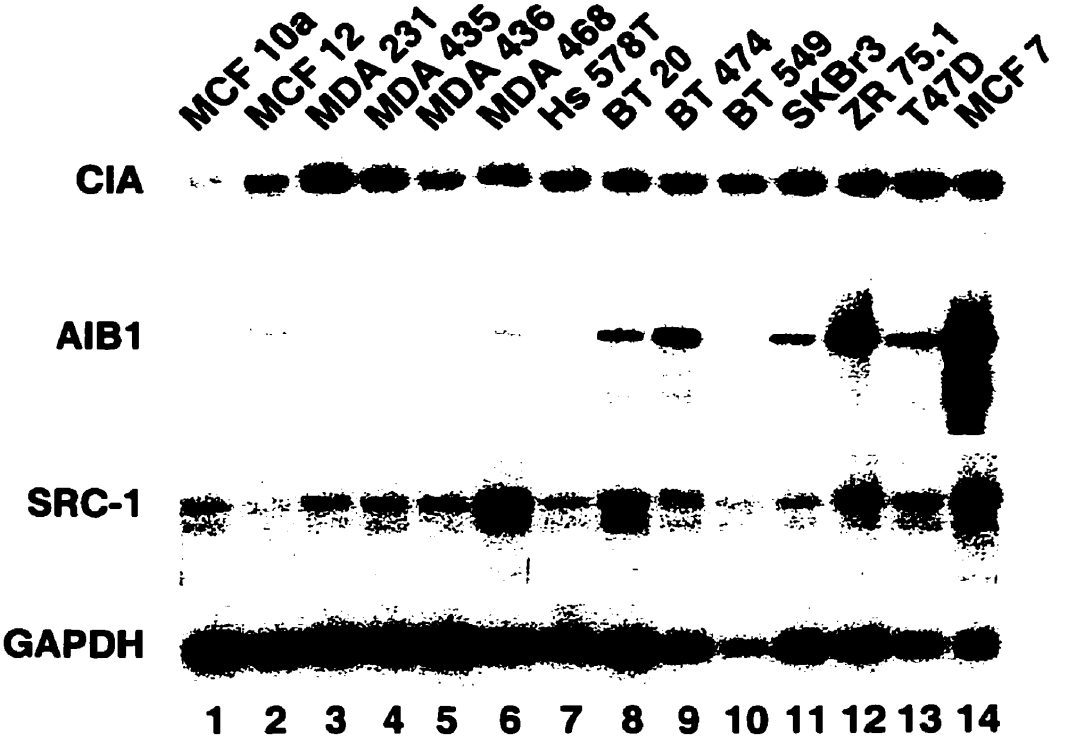

MCF 10a
MCF 12
MDA 231
MDA 435
MDA 436
MDA 468
Hs 578T
BT 20
BT 474
BT 549
SKBr3
ZR 75.1
T47D
MCF 7
CIA
AIB1
SRC-1
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 15 The CIA gene is not amplified in breast cancer cells. Genomic DNA, extracted during the RNA isolation (see figure 14), was digested with *Eco*RI, ran on an agarose gel and transferred to a nylon membrane. This membrane was probed with ^{32}P-labeled specific cDNA fragments from CIA (top panel), AIB1 (2nd panel), SRC-1 (3rd panel) and GAPDH (bottom panel, control for loading). Compared to the positive control for amplification (AIB1, 2nd panel), CIA gene is not amplified in the sampled cancer cell lines (compare top panel with 2nd panel).

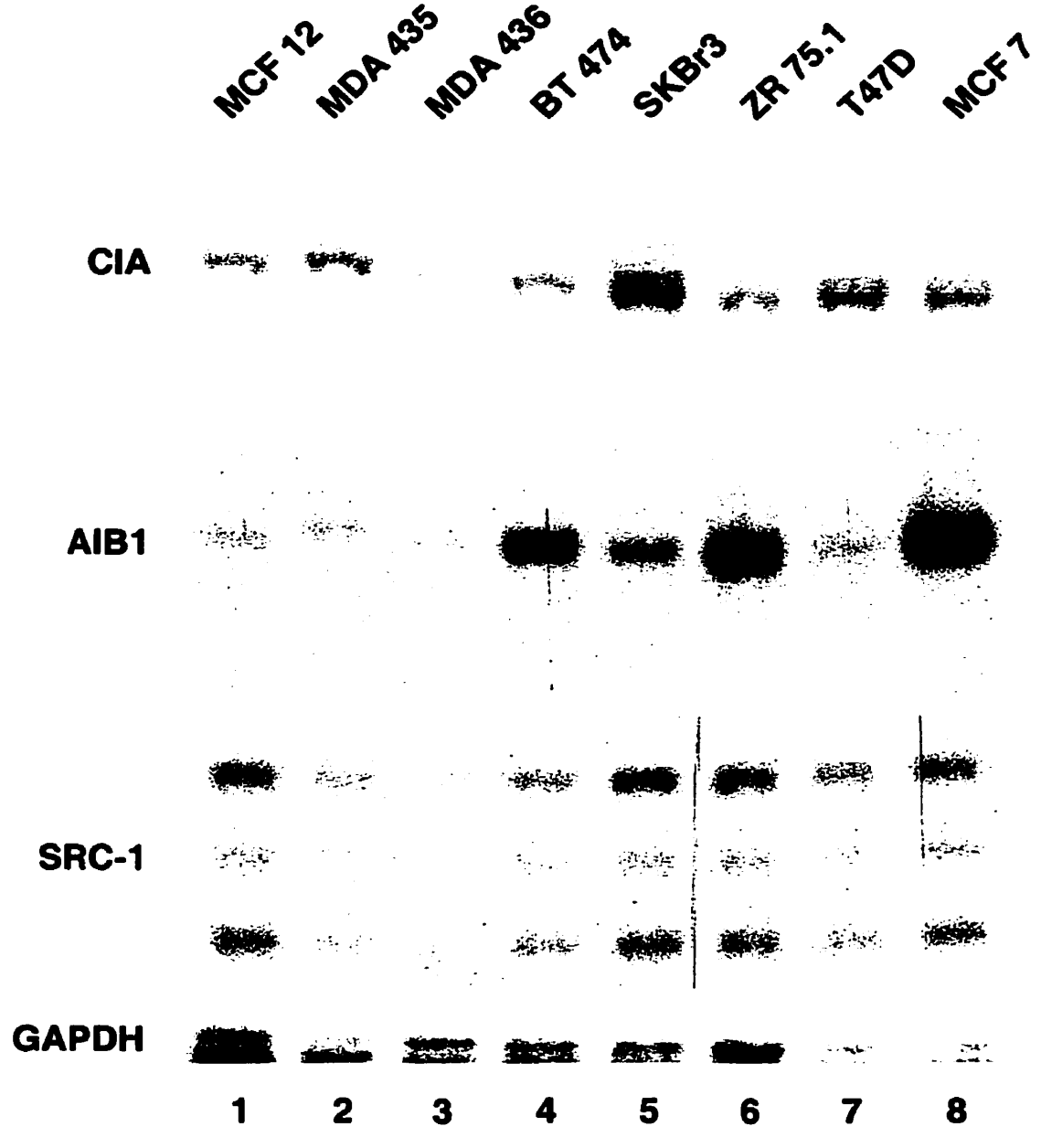

MCF 12
MDA 435
MDA 436
BT 474
SKBr3
ZR 75.1
T47D
MCF 7
CIA
AIB1
SRC-1
GAPDH
1
2
3
4
5
6
7
8

Figure 16 The residues implicated in ligand-dependent coactivator binding to the LBD of nuclear receptors. The LBD of ERα is represented here in three-dimensional rendering (modeled using Swiss PDB viewer using coordinates from the PDB file 1ERE). The 12 α-helices are in gray with the helix 12 on the top left of the image. The residues forming the hydrophobic cleft are in orange and those forming the signature motif are in yellow. Note that the orange residues are all facing the solvent and are therefore likely to be directly involved in the interaction with coactivators.

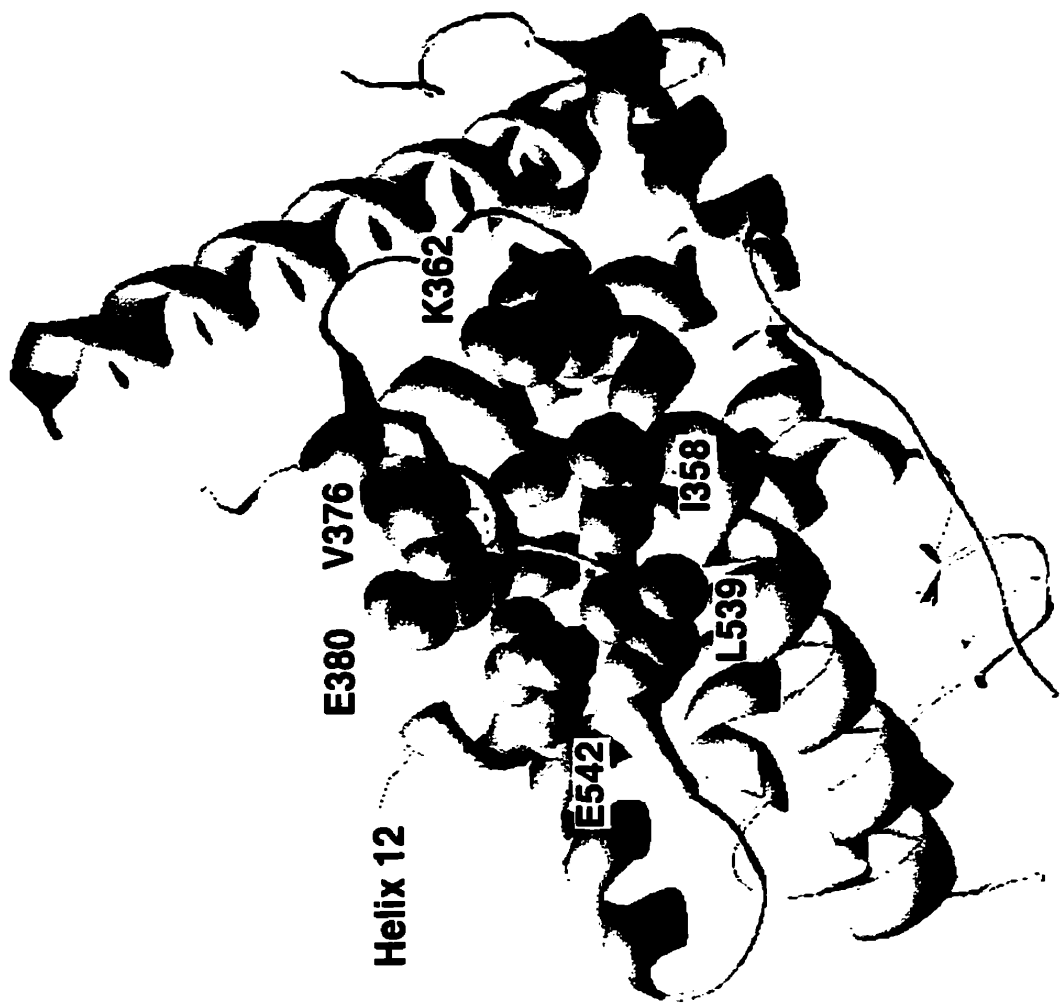

K362
V376
I358
E380
L539
E542
Helix 12

Figure 17 The residues important for ligand-dependent binding of CIA to ERα LBD are not located on the AF-2. On the same three dimensional representation of ERα LBD as seen in figure 16, is a display of the various mutations that were presented in this work. The mutated residues that disrupt the interaction with CIA are represented in red while those that still interacted with CIA are in green. Note that the residues located on the helix 12 are not necessary for the ER-CIA interaction. This view of ERα LBD provides structural information on the surface that is contacted by CIA. Based on this information, we propose that CIA binds to the LBD on the opposite side of the cleft (to the right of the drawing), and does not contact directly the AF-2.

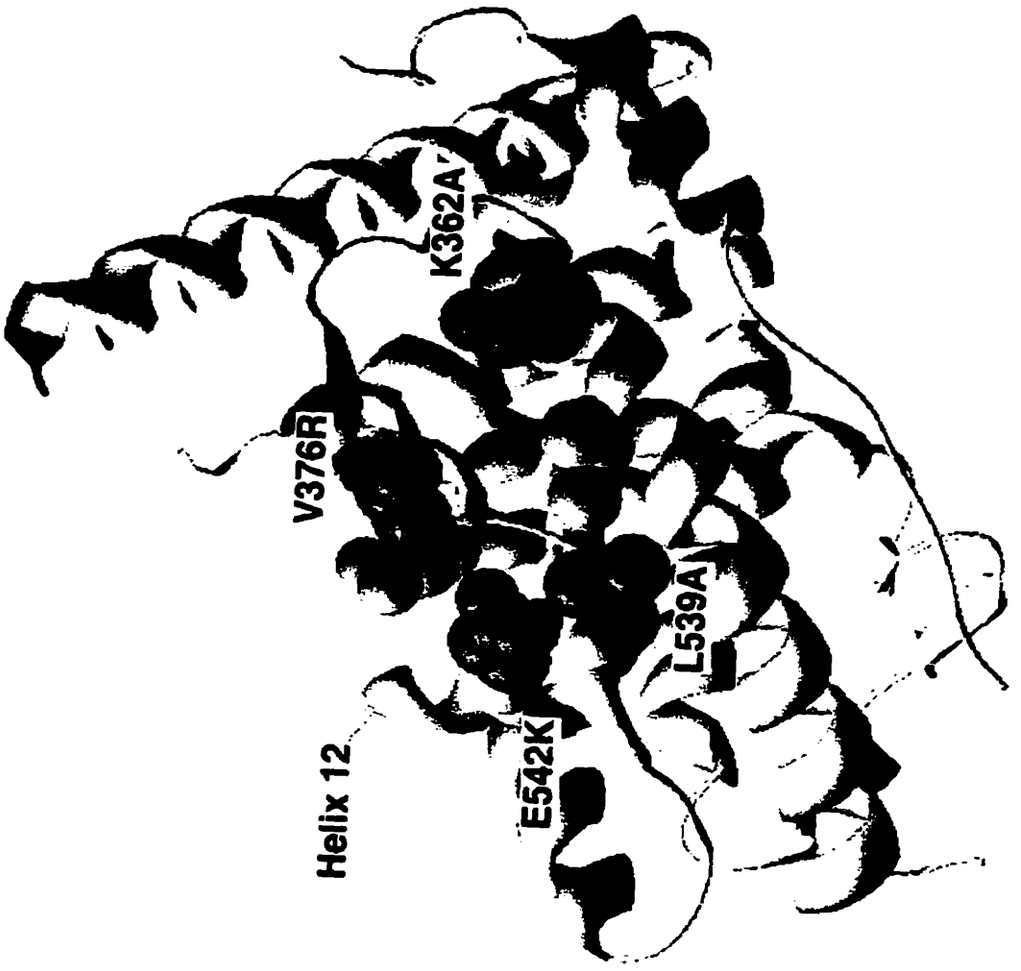

K362A
V376R
L539A
E542K
Helix 12

Table 1 Nuclear receptor coactivators

Coactivator	Comments	References
ADA	Coactivates RXR and ER, yeast acetylase complex	(vom Baur et al., 1998)
ARAs	Family of AR specific coactivators, ARA70 enhances agonist activity of partial agonists in prostate cancers cells, it also coactivates PPARγ	(Yeh and Chang, 1996)
ARIP3	Coactivates AR, only expressed in testis	(Moilanen et al., 1999)
ASC-1	Coactivates RXR, RAR, ER and TR, interacts with SRC-1 and CBP, localizes to the cytoplasm under conditions of serum deprivation	(Kim et al., 1999)
BRG-1	Coactivates GR and ER, required for GR chromatin remodeling	(Muchardt and Yaniv, 1993)
CARM1	Coactivates AR, TR and ER via binding to SRC-1, methylates histone H3, contains a SAM binding-domain	(Chen et al., 1999)
CBP	Coactivates CREB transcription factor, NR and the general activators NF-κB and p53, interacts with P/CAF, SRC-1, SRC-2 and SRC-3, HAT activity, mutated in Rubinstein/Taybi syndrome	(Kwok et al., 1994)
E6-AP	Coactivates AR, ER, PR and GR, functions as an E3 ubiquitin ligase	(Nawaz et al., 1999)
ERAP 140	Coactivates ER in a ligand-dependent manner	(Halachmi et al., 1994)
ERAP 160	Coactivates ER and GR in a ligand-dependent manner	(Halachmi et al., 1994)
HMG-1	Coactivates steroid receptors, promotes DNA binding	(Boonyarata nakornkit et al., 1998)

L7/SPA	Coactivates PR bound to the partial antagonist RU486	(Jackson et al., 1997)
NCoA-62	Coactivates ER, RAR, GR and VDR	(Baudino et al., 1998)
NIRF3	Coactivates RXR and TR	(Li et al., 1999)
NSD-1	Coactivates NR AF-2 ligand-independently, contains a SET-domain	(Huang et al., 1998)
p/CAF	Coactivates PR and TR, interacts with SRC-1 and ACTR, interacts with CBP/p300, HAT activity	(Yang et al., 1996)
p300	Resembles CBP functionally but is not functionally redundant, associates with the adenovirus E1A protein, coactivates NR, interacts with SRC-1 and SRC-3, HAT activity	(Eckner et al., 1994)
p68 RNA helicase	Coactivates ERα, specific for AF-1, interaction dependent on S118 phosphorylation, interacts with CBP	(Endoh et al., 1999)
PBP	Coactivates PPARγ and ERα, amplified and overexpressed in breast cancers	(Zhu et al., 1997)
PGC-1	Coactivates PPARγ , expressed in brown adipose tissue and skeletal muscle, induced at low temperatures	(Puigserver et al., 1998)
Positive Cofactor	Synergize with TRAPs to activate TR *in vitro*	(Fondell et al., 1999)
RIP 140	Coactivates ER, corepressor for TR2 orphan receptor	(Cavaillès et al., 1995)
SMCC	Complex homologous to TRAPs / DRIPs	(Gu et al., 1999)
SNURF	Coactivates AR, PR, GR, Sp1 and AP1	(Moilanen et al., 1998)
SRA	Coactivates steroid hormone receptors, functionally active as an RNA, specific for AF-1	(Lanz et al., 1999)

SRC-1	Coactivates NRs, interacts with CBP, p300 and P/CAF, contacts basal transcription factors, HAT activity, genetic ablation causes partial hormone insensitivity in mice	(Oñate et al., 1995)
SRC-2	Coactivates NRs, interacts with CBP	(Hong et al., 1996),
SRC-3	Coactivates NR, interacts with CBP and p300, HAT activity, overexpressed in breast and ovarian cancers	(Torchia et al., 1997),
TIF-1	Coactivates RAR/RXR in yeast, represses when fused to DNA-binding domain, interacts with chromatin remodeling complexes, protein kinase, contains RING PHD and b-box domains	(Le Douarin et al., 1995)
TIP 60	Coactivates AR, ER and PR, coactivates also the TAT protein of HIV	(Brady et al., 1999)
TRAPS / DRIPS	Coactivates TR and VDR, protein complexes of more then 10 components, similarities with the SMCC complex	(Rachez et al., 1998).
rip-1	Coactivates TR, RXR, RAR and Gal4 and VP16 in yeast, substitutes for Sug1 in yeast, contains ATPase domain	(Lee et al., 1995)
TSC-2	Coactivates VDR and PPARγ , associated with familial tuberous sclerosis	(Henry et al., 1998)

REFERENCES:

Ait-Si-Ali, S., Ramirez, S., Barre, F.-X., Dkhissi, F., Magnaghi-Jaulin, L., Girault, J. A., Robin, P., Knibiehler, M., Pritchard, L. L., Ducommun, B., Trouche, D., and Harel-Bellan, A. (1998). Histone acetyltransferase activity of CBP is controlled by cycle-dependent kinases and oncoprotein E1A. Nature *396*, 184-186.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389-3402.

Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X.-Y., Sauter, G., Kallioniemi, O.-P., Trent, J. M., and Meltzer, P. S. (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science *277*, 965-968.

Aronica, S. M., and Katzenellenbogen, B. S. (1993). Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. Mol. Endocrinol. *7*, 743-752.

Avantaggiati, M. L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A. S., and Kelly, K. (1997). Recruitment of p300/CBP in p53-dependent signal pathways. Cell *89*, 1175-84.

Bannister, A. J., and Kouzarides, T. (1996). The CBP co-activator is a histone acetyltransferase. Nature *384*, 641-643.

Baudino, T. A., Kraichely, D. M., Jefcoat, S. C., Jr., Winchester, S. K., Partridge, N. C., and MacDonald, P. N. (1998). Isolation and characterization of a novel coactivator protein, NCoA-62, involved in vitamin D-mediated transcription. J Biol Chem *273*, 16434-41.

Bautista, S., Valles, H., Walker, R. L., Anzick, S., Zeillinger, R., Meltzer, P., and Theillet, C. (1998). In breast cancer, amplification of the steroid receptor coactivator gene AIB1 is correlated with estrogen and progesterone receptor positivity. Clin. Cancer Res. *4*, 2925-2929.

Beato, M., Herrlich, P., and Schütz, G. (1995). Steroid hormone receptors: many actors in search of a plot. Cell *83*, 851-857.

Beato, M., and Sánchez-Pacheco, A. (1996). Interaction of steroid hormone receptors with the transcription initiation complex. Endocr. Rev. *17*, 587-609.

Berkenstam, A., Vivanco Ruiz, M. d. M., Barettino, D., Horikoshi, M., and Stunnenberg, H. G. (1992). Cooperativity in transcription between retinoic acid receptor and TFIID requires an activity analogous to E1A. Cell *69*, 401-412.

Blanco, J. C., Wang, I. M., Tsai, S. Y., Tsai, M. J., O'Malley, B. W., Jurutka, P. W., Haussler, M. R., and Ozato, K. (1995). Transcription factor TFIIB and the vitamin D receptor cooperatively activate ligand-dependent transcription. Proc Natl Acad Sci U S A *92*, 1535-9.

Blumberg, B., and Evans, R. M. (1998). Orphan nuclear receptors-new ligands and new possibilities. Genes Dev. *12*, 3149-3155.

Boonyaratanakornkit, V., Melvin, V., Prendergast, P., Altmann, M., Ronfani, L., Bianchi, M. E., Taraseviciene, L., Nordeen, S. K., Allegretto, E. A., and Edwards, D. P. (1998). High-mobility group chromatin proteins 1 and 2 functionally interact with steroid hormone receptors to enhance their DNA binding in vitro and transcriptional activity in mammalian cells. Mol Cell Biol *18*, 4471-87.

Brady, M. E., Ozanne, D. M., Gaughan, L., Waite, I., Cook, S., Neal, D. E., and Robson, C. N. (1999). Tip60 is a nuclear hormone receptor coactivator. J Biol Chem *274*, 17599-604.

Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996). Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell *84*, 843-51.

Brzozowski, A. M., Pike, A. C. W., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, L., Greene, G. L., Gustafsson, J.-Å., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature *389*, 753-758.

Bunone, G., Briand, P.-A., Miksicek, R. J., and Picard, D. (1996). Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J. *15*, 2174-2183.

Cavaillès, V., Dauvois, S., Danielian, P. S., and Parker, M. G. (1994). Interaction of proteins with transcriptionally active estrogen receptors. Proc. Natl. Acad. Sci. USA *91*, 10009-13.

Cavaillès, V., Dauvois, S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. (1995). Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. EMBO J. *14*, 3741-3751.

Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., Montminy, M., and Evans, R. M. (1996). Role of CBP/P300 in nuclear receptor signalling. Nature *383*, 99-103.

Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S. M., Schurter, B. T., Aswad, D. W., and Stallcup, M. R. (1999). Regulation of transcription by a protein methyltransferase. Science *284*, 2174-2177.

Chen, H., Lin, R. J., Schlitz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997). Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and form a multimeric activation complex with P/CAF and CBP/p300. Cell *90*, 569-580.

Chen, H., Lin, R. J., Xie, W., Wilpitz, D., and Evans, R. M. (1999). Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. Cell *98*, 675-686.

Coleman, R. A., Taggart, A. K. P., Burma, S., Chicca II, J. J., and Pugh, B. F. (1999). TFIIA regulates TBP and TFIID dimers. Mol. Cell *4*, 451-457.

Couse, J. F., and Korach, K. S. (1999). Estrogen receptor null mice: what have we learned and where will they lead us? Endocr. Rev. *20*, 358-417.

Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992). Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. EMBO J. *11*, 1025-1033.

Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998). Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev. *12*, 3343-3356.

Davidson, N. E. (1998). Environmental estrogens and breast cancer risk. Curr Opin Oncol *10*, 475-8.

Doucas, V., Tini, M., Egan, D. A., and Evans, R. M. (1999). Modulation of CREB binding protein function by the promyelocytic (PML) oncoprotein suggests a role for nuclear bodies in hormone signaling. Proc. Natl. Acad. Sci. USA *96*, 2627-2632.

Eckner, R., Ewen, M. E., Newsome, D., Gerdes, M., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994). Molecular cloning and functional analysis of the adenovirus E1A- associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev *8*, 869-84.

Ellis, M. J., Jenkins, S., Hanfelt, J., Redington, M. E., Taylor, M., Leek, R., Siddle, K., and Harris, A. (1998). Insulin-like growth factors in human breast cancer. Breast Cancer Research Treatment *52*, 175-184.

Endoh, H., Maruyama, K., Masuhiro, Y., Kobayashi, Y., Goto, M., Tai, H., Yanagisawa, J., Metzger, D., Hashimoto, S., and Kato, S. (1999). Purification and identification of p68 RNA helicase acting as a transcriptional coactivator specific for the activation function 1 of human estrogen receptor alpha. Mol Cell Biol *19*, 5363-72.

Enmark, E., and Gustafsson, J. A. (1996). Orphan nuclear receptors - the first eight years. Mol. Endocrinol. *10*, 1293-1307.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240*, 889-895.

Feng, W., Ribeiro, R. C. J., Wagner, R. L., Nguyen, H., Apriletti, J. W., Fletterick, R. J., Baxter, J. D., Kushner, P. J., and West, B. L. (1998). Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science *280*, 1747-1749.

Ferguson, A. T., Lapidus, R. G., and Davidson, N. E. (1998). The regulation of estrogen receptor expression and function in human breast cancer. Cancer Treat Res *94*, 255-78.

Fondell, J. D., Ge, H., and Roeder, R. G. (1996). Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. Proc Natl Acad Sci U S A *93*, 8329-33.

Fondell, J. D., Guermah, M., Malik, S., and Roeder, R. G. (1999). Thyroid hormone receptor-associated proteins and general positive cofactors mediate thyroid hormone receptor function in the absence of the TATA box-binding protein-associated factors of TFIID. Proc. Natl. Acad. Sci. USA *96*, 1959-1964.

Fornander, T., Rutquist, L. E., Cedermark, B., Glas, U., Mattson, A., Siljverward, J. D., Skoog, L., Somell, A., Theve, T., Wilking, N., Askergren, J., and Hjolmar, M. L. (1989). Adjuvant tamoxifen in early breast cancer: occurrence of new primary cancers. Lancet *1*, 117-120.

Freemont, P. S. (1993). The RING finger. A novel protein sequence motif related to the zinc finger. Ann N Y Acad Sci *684*, 174-92.

Fronsdal, K., Engedal, N., Slagsvold, T., and Saatcioglu, F. (1998). CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1. J Biol Chem *273*, 31853-9.

Fujimoto, N., Yeh, S., Kang, H. Y., Inui, S., Chang, H. C., Mizokami, A., and Chang, C. (1999). Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. J. Biol. Chem. *274*, 8316-8321.

Gallant, J. (1997). Identification of a Novel Gene Product Interacting with the Orphan Nuclear Receptors RVR/RevErba Using the Yeast Two-Hybrid System: McGill University).

Ghadimi, B. M., Schrock, E., Walker, R. L., Wangsa, D., Jauho, A., Meltzer, P. S., and Ried, T. (1999). Specific chromosomal aberrations and amplification of the AIB1 nuclear receptor coactivator gene in pancreatic carcinomas. American Journal of Pathology *154*, 525-536.

Giguère, V. (1999). Orphan nuclear receptors: from gene to function. Endocr. Rev. *20*, in press.

Giguère, V. (1994). Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. Endocr. Rev. *15*, 61-79.

Giguère, V., Hollenberg, S. H., Rosenfeld, M. G., and Evans, R. M. (1986). Functional domains of the human glucocorticoid receptor. Cell *46*, 645-652.

Giguère, V., McBroom, L. D. B., and Flock, G. (1995). Determinants of target gene specificity for RORα1: monomeric DNA-binding by an orphan nuclear receptor. Mol. Cell. Biol. *15*, 2517-2526.

Giguère, V., Tini, M., Flock, G., Ong, E. S., Evans, R. M., and Otulakowski, G. (1994). Isoform-specific amino-terminal domains dictate DNA-binding properties of RORα, a novel family of orphan nuclear receptors. Genes Dev. *8*, 538-553.

Giguère, V., Tremblay, A., and Tremblay, G. B. (1998). Estrogen receptor β: re-evaluation of estrogen and antiestrogen signaling. Steroids *63*, 335-339.

Glass, C. K. (1994). Differential recognition of target genes by nuclear receptors monomers, dimers, and heterodimers. Endocr. Rev. *15*, 391-407.

Green, S., Kumar, V., Theulaz, I., Wahli, W., and Chambon, P. (1988). The N-terminal DNA-binding 'zinc finger' of the oestrogen and glucocorticoid receptors determines target gene specificity. EMBO J. *7*, 3047-3044.

Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., and Shine, J. (1986). Sequence and expression of human estrogen receptor complementary DNA. Science *231*, 1150-1154.

Grese, T. A., Sluka, J. P., Bryant, H. U., Cullinan, G. J., Glasebrook, A. L., Jones, C. D., Matsumoto, K., Palkowitz, A. D., Sato, M., Termine, J. D., Winter, M. A., Yang, N. A., and Dodge, J. A. (1997). Molecular determinants of tissue selectivity in estrogen receptor modulators. Proc. Natl. Acad. Sci. USA *94*, 14105-14110.

Gu, W., Malik, S., Ito, M., Yuan, C. X., Fondell, J. D., Zhang, X., Martinez, E., Qin, J., and Roeder, R. G. (1999). A novel human SRB/MED-containing cofactor complex, SMCC, involved in transcription regulation. Mol. Cell *3*, 97-108.

Guan, X. Y., Xu, J., Anzick, S. L., Zhang, H., Trent, J. M., and Meltzer, P. S. (1996). Hybrid selection of transcribed sequences from microdissected DNA: isolation of genes within amplified region at 20q11-q13.2 in breast cancer. Cancer Res. *56*, 3446-3450.

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

Ha, I., Roberts, S., Maldonado, E., Sun, X., Kim, L. U., Green, M., and Reinberg, D. (1993). Multiple functional domains of human transcription factor IIB: distinct interactions with two general transcription factors and RNA polymerase II. Genes Dev *7*, 1021-32.

Hadzic, E., Desai-Yajnik, V., Helmer, E., Guo, S., Wu, S., Koudinova, N., Casanova, J., Raaka, B. M., and Samuels, H. H. (1995). A 10-amino-acid sequence in the N-terminal A/B domain of thyroid hormone receptor alpha is essential for transcriptional activation and interaction with the general transcription factor TFIIB. Mol Cell Biol *15*, 4507-17.

Halachmi, S., Marden, E., Martin, G., MacKay, H., Abbondanza, C., and Brown, M. (1994). Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. Science *264*, 1455-1458.

Hankinson, O. (1995). The aryl hydrocarbon receptor complex. Annu Rev Pharmacol Toxicol *35*, 307-40.

Harding, H. P., and Lazar, M. A. (1993). The orphan receptor Rev-ErbAα activates transcription via a novel response element. Mol. Cell. Biol. *13*, 3113-3121.

Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature *387*, 733-736.

Heinlein, C. A., Ting, H. J., Yeh, S., and Chang, C. (1999). Identification of ARA70 as a ligand-enhanced coactivator for the peroxisome proliferator-activated receptor γ. J. Biol. Chem. *274*, 16147-16152.

Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997). A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. Nature *387*, 43-48.

Henikoff, S., and Henikoff, J. G. (1994). Protein family classification based on searching a database of blocks. Genomics *19*, 97-107.

Henry, K. W., Yuan, X., Koszewski, N. J., Onda, H., Kwiatkowski, D. J., and Noonan, D. J. (1998). Tuberous sclerosis gene 2 product modulates transcription mediated by steroid hormone receptor family members. J Biol Chem *273*, 20535-9.

Hong, H., Kohli, K., Trivedi, A., Johnson, D. L., and Stallcup, M. R. (1996). GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. Proc. Natl. Acad. Sci. USA *93*, 4948-4952.

Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamel, Y., Soderstrom, M., Glass, C. K., and Rosenfeld, M. G. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature *377*, 397-404.

Horwitz, K. B., Jackson, T. A., Rain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996). Nuclear receptor coactivators and corepressors. Mol. Endocrinol. *10*, 1167-1177.

Hsiao, P. W., and Chang, C. (1999). Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells. J Biol Chem *274*, 22373-9.

Hsiao, P. W., Lin, D. L., Nakao, R., and Chang, C. (1999). The linkage of Kennedy's neuron disease to ARA24, the first identified androgen receptor polyglutamine region-associated coactivator. J Biol Chem *274*, 20229-34.

Huang, N., vom Baur, E., Garnier, J. M., Lerouge, T., Vonesch, J. L., Lutz, Y., Chambon, P., and Losson, R. (1998). Two distinct nuclear receptor interaction domains in NSD1, a novel SET protein that exhibits characteristics of both corepressors and coactivators. Embo J *17*, 3398-412.

Ikonen, T., Palvimo, J. J., and Janne, O. A. (1997). Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. J Biol Chem *272*, 29821-8.

Ing, N. H., Beekman, J. M., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1992). Members of the steroid hormone receptor superfamily interact with TFIIB (S300-II). J Biol Chem *267*, 17617-23.

Jackson, T. A., Richer, J. K., Bain, D. L., Takimoto, G. S., Tung, L., and Horwitz, K. B. (1997). The Partial Agonist Activity Of Antagonist-Occupied Steroid Receptors Is Controlled By a Novel Hinge Domain-Binding Coactivator L7/Spa and the Corepressors N-Cor or Smrt. Mol. Endocrinol. *11*, 693-705.

Jacq, X., Brou, C., Lutz, Y., Davidson, I., Chambon, P., and Tora, L. (1994). Human $TAF_{II}30$ is present in a disctinct TFIID complex and is required for transcriptional activation by the estrogen receptor. Cell *79*, 107-117.

Janknecht, R., and Hunter, T. (1996). A growing coactivator network. Nature *383*, 22-23.

Jenster, G., Spencer, T. E., Burcin, M. M., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997). Steroid receptor induction of gene transcription: a two-step model. Proc Natl Acad Sci U S A *94*, 7879-84.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.-C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996). A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell *85*, 403-414.

Kang, H. Y., Yeh, S., Fujimoto, N., and Chang, C. (1999). Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. J. Biol. Chem. *274*, 8570-8576.

Kastner, P., Mark, M., and Chambon, P. (1995). Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? Cell *83*, 859-869.

Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., Metzger, D., and Chambon, P. (1995). Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science *270*, 1491-1494.

Kim, H. J., Yi, J. Y., Sung, H. S., Moore, D. D., Jhun, B. H., Lee, Y. C., and Lee, J. W. (1999). Activating signal cointegrator 1, a novel transcription coactivator of nuclear receptors, and its cytosolic localization under conditions of serum deprivation. Mol Cell Biol *19*, 6323-32.

Kim, H.J., and Cochran, B.H. (2000). Extracellular signal-regulated kinase binds to TFII-I and regulates its activation on the c-fos promoter. Mol Cell Biol *20*, 1140-48.

Klein, C., and Struhl, K. (1994). Increased recruitment of TATA-binding protein to the promoter by transcriptional activation domains in vivo. Science *266*, 280-2.

Kliewer, S. A., Lehmann, J. M., and Willson, T. M. (1999). Orphan nuclear receptors: shifting endocrinology into reverse. Science *284*, 757-760.

Kliewer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Watson, M. A., Jones, S. A., McKee, D. D., Oliver, B. B., Wilson, T. M., Zetterström, R. H., Perlmann, T., and Lehmann, J. M. (1998). An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell *92*, 73-82.

Koleske, A. J., and Young, R. A. (1994). An RNA polymerase II holoenzyme responsive to activators [see comments]. Nature *368*, 466-9.

Kozak, M. (1992). Regulation of translation in eukaryotic systems. Ann. Rev. Cell Biol. *8*, 197-225.

Kuiper, G. G. J. M., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J.-Å. (1996). Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl. Acad. Sci. USA *93*, 5925-5930.

Kwok, R. P. S., Lundblad, J. R., Chrivla, J. C., Richards, J. P., Bächlnger, H. P., Brennan, R. G., Roberts, S. G. E., Green, M. R., and Goodman, R. H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature *370*, 223-226.

Kwon, H., Imbalzano, A. N., Khavari, P. A., Kingston, R. E., and Green, M. R. (1994). Nucleosome disruption and enhancement of activator binding by a human SW1/SNF complex [see comments]. Nature *370*, 477-81.

Laherty, C. D., Yang, W. M., Sun, J. M., Davie, J. R., Seto, E., and Eisenman, R. N. (1997). Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. Cell *89*, 349-56.

Lanz, R. B., McKenna, N. J., Onate, S. A., Albrecht, U., Wong, J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1999). A steroid receptor coactivator, SRA, functions as an RNA and is present in an SRC-1 complex. Cell *97*, 17-27.

Lavigne, A. C., Mengus, G., Gangloff, Y. G., Wurtz, J. M., and Davidson, I. (1999). Human TAF(II)55 interacts with the vitamin D(3) and thyroid hormone receptors and with derivatives of the retinoid X receptor that have altered transactivation properties. Mol Cell Biol *19*, 5486-94.

Le Douarin, B., Nielsen, A. L., Garnier, J.-M., Ichinose, H., Jeanmougin, F., Losson, R., and Chambon, P. (1996). A possible involvement of TIF1α and TIF1β in the epigenic control of transcription by nuclear receptors. EMBO J. *15*, 6701-6715.

Le Douarin, B., You, J., Nielsen, A. L., Chambon, P., and Losson, R. (1998). TIF1alpha: a possible link between KRAB zinc finger proteins and nuclear receptors. J Steroid Biochem Mol Biol *65*, 43-50.

Le Douarin, B., Zechel, C., Garnier, J.-M., Lutz, Y., Tora, L., Pierrat, B., Heery, D., Gronemeyer, H., Chambon, P., and Losson, R. (1995). The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. EMBO J. *14*, 2020-2033.

Leblanc, B. P., and Stunnenberg, H. G. (1995). 9-cis retinoic acid signaling: changing partners causes some excitement. Genes Dev. *9*, 1811-1816.

Lee, C. H., Chinpaisal, C., and Wei, L. N. (1998). Cloning and characterization of mouse RIP140, a corepresor for nuclear orphan receptor TR2. Mol Cell Biol *18*, 6745-55.

Lee, J. W., Ryan, F., Swaffield, J. C., Johnston, S. A., and Moore, D. D. (1995). Interaction of thyroid-hormone receptor with a conserved transcriptional mediator. Nature *374*, 91-94.

Lee, S., and Hahn, S. (1995). Model for binding of transcription factor TFIIB to the TBP-DNA complex. Nature *376*, 609-12.

Leygue, E., Dotzlaw, H., Watson, P. H., and Murphy, L. C. (1998). Altered estrogen receptor α and β messenger RNA expression during human breast tumorigenesis. Cancer Res. *58*, 3197-3201.

Li, D., Desai-Yajnik, V., Lo, E., Schapira, M., Abagyan, R., and Samuels, H. H. (1999). NRIF3 Is a Novel Coactivator Mediating Functional Specificity of Nuclear Hormone Receptors. Mol Cell Biol *19*, 7191-7202.

Li, H., and Chen, J. D. (1998). The receptor-associated coactivator 3 activates transcription through CREB-binding protein recruitment and autoregulation. J. Biol. Chem. *273*, 5948-5954.

Li, H., Gomes, P. J., and Chen, J. D. (1997). RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. Proc. Natl. Acad. Sci. USA *94*, 8479-8484.

Li, Q., Imhof, A., Collingwood, T. N., Urnov, F. D., and Wolffe, A. P. (1999). p300 stimulates transcription instigated by ligand-bound thyroid hormone receptor at a step subsequent to chromatin disruption. EMBO J. *18*, 5634-5652.

Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251-260.

MacGregor, J. I., and Jordan, V. C. (1998). Basic guide to the mechanisms of antiestrogen action. Pharmacology Reviews *50*, 151-196.

Makela, S., Savolainen, H., Aavik, E., Myllarniemi, M., Strauss, L., Taskinen, E., Gustafsson, J. A., and Hayry, P. (1999). Differentiation between vasculoprotective and uterotrophic effects of ligands with different binding affinities to estrogen receptors alpha and beta. Proc. Natl. Acad. Sci. USA *96*, 7077-7782.

Mangelsdorf, D. J., and Evans, R. M. (1995). The RXR heterodimers and orphan receptors. Cell *83*, 841-850.

Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995). The nuclear receptor superfamily: the second decade. Cell *83*, 835-839.

May, M., Mengus, G., Lavigne, A. C., Chambon, P., and Davidson, I. (1996). Human TAF(II28) promotes transcriptional stimulation by activation function 2 of the retinoid X receptors. Embo J *15*, 3093-104.

McBroom, L. D. B., Flock, G., and Giguère, V. (1995). The non-conserved hinge region and distinct amino-terminal domains of the RORα orphan nuclear receptor isoforms are required for proper DNA bending and RORα-DNA interactions. Mol. Cell. Biol. *15*, 796-808.

McEwan, I. J., and Gustafsson, J.-Å. (1997). Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF. Proc. Natl. Acad. Sci. USA *94*, 8485-8490.

McInerney, E. M., Rose, D. W., Flynn, S. E., Westin, S., Mullen, T. M., Krones, A., Inostroza, J., Torchia, J., Nolte, R. T., Assa-Munt, N., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1998). Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. Genes Dev. *12*, 3357-3368.

McInerney, E. M., Tsai, M.-J., O'Malley, B. W., and Katzenellenbogen, B. S. (1996). Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator. Proc. Natl. Acad. Sci. USA *93*, 10069-10073.

McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999). Nuclear receptor coregulators: cellular and molecular biology. Endocr. Rev. *20*, 321-344.

McKenna, N. J., Nawaz, Z., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1998). Distinct steady-state nuclear receptor coregulator complexes exist in vivo. Proc Natl Acad Sci U S A *95*, 11697-702.

Mengus, G., May, M., Carre, L., Chambon, P., and Davidson, I. (1997). Human TAF(II)135 potentiates transcriptional activation by the AF-2s of the retinoic acid, vitamin D3, and thyroid hormone receptors in mammalian cells. Genes Dev *11*, 1381-95.

Mengus, G., May, M., Jacq, X., Staub, A., Tora, L., Chambon, P., and Davidson, I. (1995). Cloning and characterization of hTAFII18, hTAFII20 and hTAFII28: three subunits of the human transcription factor TFIID. Embo J *14*, 1520-31.

Meyer, M.-E., Gronemeyer, H., Turcotte, B., Bocquel, M.-T., Tasset, D., and Chambon, P. (1989). Steroid hormone receptors compete for factors that mediate their enhancer function. Cell *57*, 433-442.

Miyamoto, H., Yeh, S., Wilding, G., and Chang, C. (1998). Promotion of agonist activity of antiandrogens by the androgen receptor coactivator, ARA70, in human prostate cancer DU145 cells. Proc Natl Acad Sci U S A *95*, 7379-84.

Mizzen, C. A., Yang, X. Y., Kokubo, T., Brownell, J. E., Bannister, A. J., Owen-Hughes, T., Workman, J., Wang, L., Berger, S. L., Kouzarides, T., Nakatani, Y., and Allis, C. D. (1996). The $TAF_{II}250$ subunit of TFIID has histone acetyltransferase activity. Cell *87*, 1261-70.

Moilanen, A. M., Karvonen, U., Poukka, H., Yan, W., Toppari, J., Jänne, O. A., and Palvimo, J. J. (1999). A testis-specific androgen receptor coregulator that belongs to a novel family of nuclear proteins. J. Biol. Chem. *274*, 3700-3704.

Moilanen, A. M., Poukka, H., Karvonen, U., M, H. k., OA, J. n., and Palvimo, J. J. (1998). Identification of a Novel RING Finger Protein as a Coregulator in Steroid Receptor-Mediated Gene Transcription. Mol Cell Biol *18*, 5128-39.

Moosmann, P., Georgiev, O., Le Douarin, B., Bourquin, J. P., and Schaffner, W. (1996). Transcriptional repression by RING finger protein TIF1 beta that interacts with the KRAB repressor domain of KOX1. Nucleic Acids Res *24*, 4859-67.

Mosselman, S., Polman, J., and Dijkema, R. (1996). ERβ: identification and characterization of a novel human estrogen receptor. FEBS Letters *392*, 49-53.

Muchardt, C., and Yaniv, M. (1993). A human homologue of Saccharomyces cerevisiae SNF2/SWI2 and Drosophila brm genes potentiates transcriptional activation by the glucocorticoid receptor. Embo J *12*, 4279-90.

Murphy, L. C., Dotzlaw, H., Leygue, E., Coutts, A., and Watson, P. (1998). The pathophysiological role of estrogen receptor variants in human breast cancer. J Steroid Biochem Mol Biol *65*, 175-80.

Mytelka, D. S., and Chamberlin, M. J. (1996). Analysis and suppression of DNA polymerase pauses associated with a trinucleotide consensus. Nucleic Acid Research *24*, 2774-2781.

Nardulli, A. M., and Shapiro, D. J. (1992). Binding of the estrogen receptor DNA-binding domain to the estrogen response element induces DNA bending. Mol. Cell. Biol. *12*, 2037-2042.

Nawaz, Z., Lonardt, D. M., Smith, C. L., Lev-Lehman, E., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1999). The Angelman syndrome-associated protein, E6-AP, is a

coactivator for the nuclear hormone receptor superfamilly. Molecular and Cellular Biology *19*, 1182-1189.

Neely, K. E., Hassan, A. H., Wallberg, A. E., Steger, D. J., Cairns, B. R., Wright, A. P. H., and Workman, J. L. (1999). Activation domain-mediated targeting of the SWI/SNF complex to promoters stimulates transcription from nucleosome arrays. Mol. Cell *4*, 649-655.

Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V. (1998). Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-γ. Nature *395*, 137-143.

Nuclear receptor nomenclature committee, T. n. r. n. (1999). A unified nomenclature system for the nuclear receptors superfamily. Cell *97*, 161-163.

Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell *87*, 953-959.

O'Malley, B. W., and Conneley, O. M. (1992). Orphan receptors: in search of a unifying hypothesis for activation. Mol. Endocrinol. *6*, 1359-1361.

Oñate, S. A., Boonyaratanakornkit, V., Spencer, T. E., Tsai, S. Y., Tsai, M. J., Edwards, D. P., and O'Malley, B. W. (1998). The steroid receptor coactivator-1 contains multiple receptor interacting and activation domains that cooperatively enhance the activation function 1 (AF1) and AF2 domains of steroid receptors. J. Biol. Chem. *273*, 12101-12108.

Oñate, S. A., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1995). Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science *270*, 1354-1357.

Paech, K., Webb, P., Kuiper, G. G. J. M., Nilsson, S., Gustafsson, J.-Å., Kushner, P. J., and Scanlan, T. S. (1997). Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science *277*, 1508-1510.

Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997). Regulation of NF-kappaB by cyclin-dependent kinases associated with the p300 coactivator. Science *275*, 523-7.

Petrij, F., Giles, R. H., Dauwerse, H. G., Saris, J. J., Hennekam, R. C., Masuno, M., Tommerup, N., van Ommen, G. J., Goodman, R. H., Peters, D. J., and et al. (1995). Rubinstein-Taybi syndrome caused by mutations in the transcriptional co- activator CBP [see comments]. Nature *376*, 348-51.

Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M., and Spiegelman, B. M. (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell *92*, 829-839.

Rachez, C., Suldan, Z., Ward, J., Chang, C. P. B., Burakov, D., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1998). A novel protein complex that interacts with the vitamin D3 receptor in a ligand-dependent manner and enhances VDR transactivation in a cell- free system. Genes Dev. *12*, 1787-1800.

Rees, W. A., Yager, T. D., Korte, J., and von Hippel, P. H. (1993). Betaine can eliminate the base pair composition dependence of DNA melting. Biochemistry *32*, 137-144.

Rhodes, D. (1997). The nucleosome core all wrapped up. Nature *389*, 231-233.

Richmond, T. J., Finch, J. T., Rushton, B., Rhodes, D., and Klug, A. (1984). Structure of the nucleosome core particle at 7 A resolution. Nature *311*, 532-37.

Rochette-Egly, C., Adam, S., Rossignol, M., Egly, J.-M., and Chambon, P. (1997). Stimulation of RARα activation function AF-1 through binding to the general transcription factor TFIIH and phosphorylation by CDK7. Cell *90*, 97-107.

Roeder, R. G. (1996). The role of general initiation factors in transcription by RNA polymerase II. Trends Biochem Sci *21*, 327-35.

Roy, A.L., Du, H., Gregor, P.D., Novina, C.D., Martinez, E., Roeder, R.G. (1997). Cloning of an inr- and E-box binding protein TFII-I that interacts physically and functionally with USF1. Embo J *16*, 7091-104.

Rubin, D. M., Coux, O., Wefes, I., Hengartner, C., Young, R. A., Goldberg, A. L., and Finley, D. (1996). Identification of the gal4 suppressor Sug1 as a subunit of the yeast 26S proteasome. Nature *379*, 655-7.

Schulman, I. G., Chakravarti, D., Juguilon, H., Romo, A., and Evans, R. M. (1995). Interactions between the retinoid X receptor and a conserved region of the TATA-binding protein mediate hormone-dependent transactivation. Proc. Natl. Acad. Sci. USA *92*, 8288-8292.

Schwabe, J. W. R., Chapman, L., Finch, J. T., and Rhodes, D. (1993). The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell *75*, 567-578.

Shao, D., and Lazar, M. A. (1999). Modulating nuclear receptor function: may the phos be with you. J. Clin. Invest. *103*, 1617-1618.

Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell *95*, 927-937.

Shibata, H., Spencer, T. E., Onate, S. A., Jenster, G., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997). Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. Recent Prog Horm Res *52*, 141-64.

Sladek, R., and Giguère, V. (1999). Initial characterization of new orphan receptors. In Nuclear receptors: a practical approach, pp. In press.

Smith, C. L., Oñate, S. A., Tsai, M.-J., and O'Malley, B. W. (1996). CREB binding protein acts synergistically with steroid receptor coactivator-1 to enhance steroid receptor-dependent transcription. Proc. Natl. Acad. Sci. USA *93*, 8884-8888.

Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J.,Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M-J., and O'Malley, B. W. (1997). Steroid receptor coactivator-1 is a histone acetyltransferase. Nature *389*, 194-198.

Takeshita, A., Cardona, G. R., Koibuchi, N., Suen, C.-S., and Chin, W. W. (1997). TRAM-1, a novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1. J. Biol. Chem. *272*, 27629-27634.

Tanner, M. M., Tirkkonen, M., Kallioniemi, A., Collins, C., Stokke, T., Karhu, R., Kowbel, D., Shadravan, F., Hintz, M., Kuo, W. L., and et al. (1994). Increased copy number at 20q13 in breast cancer: defining the critical region and exclusion of candidate genes. Cancer Res *54*, 4257-60.

Tanner, M. M., Tirkkonen, M., Kallioniemi, A., Isola, J., Kuukasjarvi, T., Collins, C., Kowbel, D., Guan, X. Y., Trent, J., Gray, J. W., Meltzer, P., and Kallioniemi, O. P. (1996). Independent amplification and frequent co-amplification of three nonsyntenic regions on the long arm of chromosome 20 in human breast cancer. Cancer Res. *56*, 3441-3445.

Thenot, S., Henriquet, C., Rochefort, H., and Cavailles, V. (1997). Differential interaction of nuclear receptors with the putative human transcriptional coactivator hTIF1. J Biol Chem *272*, 12062-8.

Toft, D., and Gorski, J. (1966). A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci U S A *55*, 1574-81.

Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997). The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature *387*, 677-684.

Tremblay, A., Tremblay, G. B., Labrie, C., Labrie, F., and Giguère, V. (1998). EM-800, a novel antiestrogen, acts as a pure antagonist of the transcriptional functions of estrogen receptors α and β. Endocrinology *139*, 111-118.

Tremblay, A., Tremblay, G. B., Labrie, F., and Giguère, V. (1999). Ligand-independent recruitment of SRC-1 by estrogen receptor β through phosphorylation of activation function AF-1. Mol. Cell *3*, 513-519.

Tremblay, G. B., Tremblay, A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Labrie, F., and Giguère, V. (1997). Cloning, chromosomal localization and functional analysis of the murine estrogen receptor β. Mol. Endocrinol. *11*, 353-365.

Vanacker, J. M., Pettersson, K., Gustafsson, J. A., and Laudet, V. (1999). Transcriptional targets shared by estrogen receptor- related receptors (ERRs) and estrogen receptor (ER) alpha, but not by ERbeta [In Process Citation]. Embo J *18*, 4270-9.

Venturini, L., You, J., Stadler, M., Galien, R., Lallemand, V., Koken, M. H., Mattei, M. G., Ganser, A., Chambon, P., Losson, R., and de Thé, H. (1999). TIF1γ, a novel member of the transcriptional intermediary factor 1 family. Oncogene *18*, 1209-1217.

Voegel, J. J., Heine, M. J. S., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. (1998). The coactivator TIF2 contains three nuclear receptor-binding motifs and mediates transactivation through CBP binding-dependent and independent pathways. EMBO J. *17*, 507-519.

Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P., and Gronemeyer, H. (1996). TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. EMBO J. *15*, 3667-3675.

vom Baur, E., Harbers, M., Um, S. J., Benecke, A., Chambon, P., and Losson, R. (1998). The yeast Ada complex mediates the ligand-dependent activation function AF-2 of retinoid X and estrogen receptors. Genes Dev *12*, 1278-89.

von Baur, E., Zechel, C., Heery, D., Heine, M. J. S., Garnier, J. M., Vivat, V., Le Douarin, B., Gronemeyer, H., Chambon, P., and Losson, R. (1996). Differential ligand-dependent interactions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1. EMBO J. *15*, 110-124.

Walter, P., Green, S., Greene, G., Krust, A., Bornert, J. M., Jeltsch, J. M., Staub, A., Jensen, E., Scrace, G., Waterfield, M., and et al. (1985). Cloning of the human estrogen receptor cDNA. Proc Natl Acad Sci U S A *82*, 7889-93.

Wang, J. C., Stafford, J. M., and Granner, D. K. (1998). SRC-1 and GRIP1 Coactivate Transcription with Hepatocyte Nuclear Factor 4. J. Biol. Chem. *273*, 30847-30850.

Westin, S., Kurokawa, R., Nolte, R. T., Wisely, G. B., McInerney, E. M., Rose, D. W., Milburn, M. V., Rosenfeld, M. G., and Glass, C. K. (1998). Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. Nature *395*, 199-202.

Willy, P. J., and Mangelsdorf, D. J. (1998). Nuclear orphan receptors: the search for novel ligands and signaling pathways. In Hormones and Signaling, B. W. O'Malley, ed. (San Diego: Academic Press), pp. 307-358.

Wilson, T. E., Fahrner, T. J., and Milbrandt, J. (1993). The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. Mol. Cell. Biol. *13*, 5794-5804.

Wilson, T. E., Paulsen, R. E., Padgett, K. A., and Milbrandt, J. (1992). Participation of non-zinc finger residues in DNA binding by two nuclear orphan receptors. Science *256*, 107-110.

Xu, Q., Singer, R. A., and Johnston, G. C. (1995). Sug1 modulates yeast transcription activation by Cdc68. Mol Cell Biol *15*, 6025-35.

Yang, X.-J. (1998). Personal communication.

Yang, X.-J., Ogryzko, V. V., Nishikawa, J.-i., Howard, B. H., and Nakatani, Y. (1996). A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature *382*, 319-324.

Yao, T.-P., Oh, S. P., Fuchs, M., Zhou, N.-D., Ch'ng, L.-E., Newsome, D., Bronson, R. T., Li, E., Livingston, D. M., and Eckner, R. (1998). Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. Cell *93*, 361-372.

Yeh, S., and Chang, C. (1996). Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. Proc Natl Acad Sci U S A *93*, 5517-21.

Yoshinaga, S. K., Peterson, C. L., Herskowitz, I., and Yamamoto, K. R. (1992). Roles of SWI1, SWI2, and SWI3 proteins for transcriptional enhancement by steroid receptors. Science *258*, 1598-604.

Yuan, C.-X., Ito, M., Fondell, J. D., Fu, Z.-Y., and Roeder, R. G. (1998). The TRAP220 component of a thyroid hormone receptor-associated protein (TRAP) coactivator complex interacts directly with nuclear receptors in a ligand-dependent fashion. Proc. Natl. Acad. Sci. USA *95*, 7939-7944.

Zamir, I., Harding, H. P., Atkins, G. B., Hörlein, A., Glass, C. K., Rosenfeld, M. G., and Lazar, M. A. (1996). A nuclear hormone receptor corepressor mediates transcriptional silencing by receptors with distinct repression domains. Mol. Cell. Biol. *16*, 5458-5465.

Zawel, L., and Reinberg, D. (1995). Common themes in assembly and function of eukaryotic transcription complexes. Annu Rev Biochem *64*, 533-61.

Zhang, J., and Fondell, J. D. (1999). Identification of mouse TRAP100: a transcriptional coregulatory factor for thyroid hormone and vitamin D receptors [In Process Citation]. Mol Endocrinol *13*, 1130-40.

Zhu, Y., Qi, C., Calandra, C., Rao, M. S., and Reddy, J. K. (1996). Cloning and identification of mouse steroid receptor coactivator-1 (mSRC-1), as a coactivator of peroxisome proliferator-activated receptor gamma. Gene Expr *6*, 185-95.

Zhu, Y., Qi, C., Jain, S., Rao, M. S., and Reddy, J. K. (1997). Isolation and characterization of PBP, a protein that interacts with peroxisome proliferator-activated receptor. J Biol Chem *272*, 25500-6.