Nuclear Receptor Coregulators
Role of Protein-Protein Interactions and cAMP/PKA Signaling

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SCIENTIFIC ENVIRONMENTS

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LIST OF PAPERS

Paper I

Mellgren G, Børud B, Hoang T, Yri OE, Fladeby C, Lien EA, Lund J. Characterization of receptor-interacting protein RIP140 in the regulation of SF-1 responsive target genes. *Mol Cell Endocrinol.* 2003 May 30;203(1-2):91-103.

Paper II

Børud B, Hoang T, Bakke M, Jacob AL, Lund J, Mellgren G. The nuclear receptor coactivators p300/CBP/cointegrator-associated protein (p/CIP) and transcription intermediary factor 2 (TIF2) differentially regulate PKA-stimulated transcriptional activity of Steroidogenic factor 1. *Mol Endocrinol.* 2002 Apr;16(4):757-73.

Paper III

Hoang T [*], Fenne IS *, Cook C, Børud B, Bakke M, Lien EA, Mellgren G. cAMP-dependent protein kinase regulates ubiquitin-proteasome-mediated degradation and subcellular localization of the nuclear receptor coactivator GRIP1. *J Biol Chem.* 2004 Nov 19;279(47):49120-30.

Paper IV

Hoang T, Fenne IS, Johannessen M, Lien EA, Moens U and Mellgren G (2006). cAMP Response Element Binding Protein (CREB) Interacts with the Nuclear Receptor Coactivator GRIP1 and Mediates its Degradation. *Manuscript.*

* The first two authors contributed equally to this work.

ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ACTR	Activator of the thyroid and retinoic acid receptor
AD	Activation domain
AF-1	Activation function-1
AF-2	Activation function-2
AIB1	Amplified in breast cancer-1
AR	Androgen receptor
ARC	Activator-recruited cofactor
BAT	Brown adipose tissue
bHLH	Basic helix-loop-helix
cAMP	Cyclic adenosine monophosphate
CARM1	Coactivator-associated arginine methyltransferase 1
CBP	CREB-binding protein
CREB	cAMP-response element binding protein
CtBP	C-terminal binding protein
CYP17	17α-hydroxylase
CYP21	21-hydroxylase
DAX-1	Dosage sensitive sex reversal-1
DBD	DNA binding domain
DRIP	VDR-interacting protein
E6-AP	E6-Associated protein
ER	Estrogen receptor
ERKs	Extracellular signal-regulated kinases
FSH	Follicle-stimulating hormone
GCN5	General control nonderepressed 5
GR	Glucocorticoid receptor
GRIP1	GR-interacting protein 1
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HNF-4	Hepatocyte nuclear factor-4
HREs	Hormone response elements
Hsps	Heat shock proteins
LBD	Ligand-binding domain
LCoR	Ligand-dependent corepressor
LH	Lutenizing hormone
MAPKs	Mitogen-activated protein kinases
MR	Mineralcorticoid receptor
NCoA	Nuclear receptor coactivator
N-CoR	Nuclear receptor corepressor
NF-kappa B	Nuclear factor-kappa B
NR	Nuclear receptor
p300	300 KDa-protein
pAF	Proximal activation function
PAS	Per/ARNT/Sim
pCAF	p300/CBP-associated factor
p/CIP	p300/CBP cointegrator protein
PKA	cAMP-dependent protein kinase A
PPARs	Peroxisome proliferator-activated receptors
PR	Progesterone receptor
PRMT1	Protein arginine methyltransferase 1
RAC3	Receptor-associated coactivator 3
RARs	Retinoid acid receptors
RD	Repression domain
RIP140	Receptor-interacting protein 140
RXRs	Retinoid X receptor
SF-1	Steroidogenic factor-1
SMRT	Silencing mediator of retinoid and thyroid receptors
SRC	Steroid receptor coactivator
StAR	Steroidogenic acute regulatory protein
SUN-CoR	Small ubiquitous nuclear corepressor
SWI/SFN	Human homolog of BRG (Brahma (fly)-related gene 1)
TBP	TATA binding protein
TIF2	Transcription intermediary factor 2
TRs	Thyroid receptors
TRAM 1	Thyroid hormone receptor activator molecule
TRAP	TR-associated protein
TSA	Trichostatin A
VDR	Vitamin D receptor
WAT	White adipose tissue

PREFACE

Transcriptional regulation is a fundamental process for the control of cell differentiation, morphogenesis and for the development, versatility and adaptability of any organism. In eukaryotes, a superfamily of DNA sequence-specific transcriptional factors termed nuclear receptors (NRs) regulate a diverse array of genes in responses to small ligands such as natural hormones and bioreactive metabolites (retinoids, vitamin D, cholesterol metabolites, bile acids, etc). NRs are important for many aspects of human biology. They mediate the transcriptional response to many endocrine signals involved in regulation of the reproductive process, development and oxidative metabolism, as well as lipid and energy metabolism and glucose homeostasis. NRs also play significant roles in human diseases/disorders ranging from breast and prostate cancer to diabetes and obesity. The basic mechanism for regulation of gene transcription by the NR relies on the essential participation and the dynamic and combinatorial interaction of a complex and growing network of coregulator proteins (coactivators and corepressors). Over the last few years, the pivotal roles of coregulator proteins in the turning-on and -off, and especially in the fine-tuning of transcriptional activation by NRs in response to different extracellular signals have become more evident. Abundant evidence has demonstrated that not only the NRs but also their coregulator proteins are subjected to regulation through posttranslational modifications such as phosphorylation, sumoylation, ubiquitination, acetylation and methylation, as well as intracellular translocation and proteasomal degradation. It is believed that regulation of coactivator and corepressor proteins is a critical mechanism for regulation of gene transcription by NRs in response to multiple signal transduction pathways. Thus, characterization of the molecular mechanisms that regulate coregulator protein functions is essential for the understanding of the biological roles of NRs and their related disorders.

INTRODUCTION

1. Nuclear receptors

1.1. Functional and structural domains

Nuclear receptors (NRs) comprise a superfamily of ligand-inducible transcriptional factors. Members of the NR superfamily share common structural and functional domains designated as A to F (1) (Figure 1A). The N-terminal A/B region is sequence- and length-variable among the different receptor members, and contains the autonomous activation function AF-1. The C region represents the central highly conserved DNA binding domain (DBD) that consists of two zinc finger-like motifs. This domain is responsible for binding to specific target DNA elements that are known as the hormone response elements (HREs). The variable hinge domain (D) links the DBD to another conserved multifunctional C-terminal region (E) named the ligand-binding domain (LBD). The LBD encompasses the ligand-binding pocket, the second activation function (AF-2), a dimerization domain and a sequence responsible for nuclear localization. The LBDs of most NRs are formed by 12 conserved α-helices numbered from H1 to H12, where the AF-2 domain is located in the H12. These helices are folded into a three-layered antiparallel helical sandwich forming a cavity which acts as the ligand-binding pocket (reviewed in (2)). While AF-1 is found only in certain members of the NR superfamily, in particularly the steroid receptors that contain a long N-terminus, and contributes to the constitutive ligand-independent activation by the receptors, the AF-2 is highly structurally conserved and plays critical roles in the ligand-dependent activation pathway (reviewed in (3)). The C-terminal domain (F) is variable and found only in some NRs. The function of this domain is still unknown.

1.2. Subfamilies and activation mechanisms

Nuclear receptors have been subdivided into at least six subfamilies based on evolutionary analysis of their well-conserved domains, the DBD (C) and the LBD (E) (4). One large family is composed of thyroid hormone receptor (TRs), retinoid acid receptors (RARs), vitamin D receptors (VDRs), peroxisome proliferator-activated receptors (PPARs) and different orphan receptors such as RAR-related orphan receptor (RORs). The receptor for retinoid X (RXRs) together with certain orphan receptors including hepatocyte nuclear factor 4 (HNF4) comprise the second subfamily. The receptors for steroid hormones including estrogen receptor (ERs), androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR) and mineralcorticoid receptor (MR), and the highly related orphan receptors estrogen-related

receptors (ERRs) form the third subfamily. The fourth, fifth and sixth subfamilies contain orphan receptors for which no ligand has yet been discovered. These subfamilies include NGF-induced factor B (NGFI-B), steroidogenic factor 1 (SF-1) and germ cell nuclear factor (GCNF) respectively. Following this classification, a unified nomenclature system for the NR superfamily has been developed to ease understanding of newly acquired knowledge to researchers outside and within the field (5). Selected mammalian NRs with details about receptor isoforms/subtypes and their nomenclature designated by the unified nomenclature system, cognate ligands and mode of DNA binding are listed in Table 1.

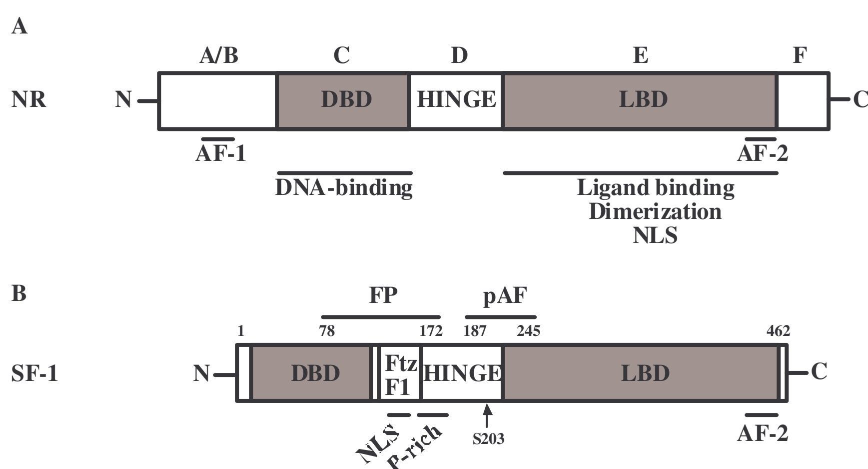

Figure 1. The functional and structural domains of a nuclear receptor. A. The common structure of a nuclear receptor: the N-terminal region (A/B) harboring a ligand-independent activation function (AF-1), the DNA binding domain (DBD, C region), the hinge or linker region (D), the ligand-binding domain (LBD, region E/F) containing the activation function 2 (AF-2), and the F region. Functions of the DBD and LBD are indicated below the bars. B. SF-1 contains all the main functional domains of a NR, including the DBD, hinge and LBD. SF-1 does not have a classical AF-1, but the FP region encompassing the fushi tarazu factor 1 (Ftz-F1) box and a proline (P)-rich region, and the proximal activation domain (pAF). NLS: nuclear localization sequence.

Table 1. Selected mammalian nuclear receptors. The subtypes and their designated nomenclature, cognate ligands and mode of binding to target gene promoter of each NR are included (1,5,6).

Receptor name	Abbreviation and subtype	Nomenclature	Ligand	DNA-binding
Thyroid hormone receptor	TRα, β	NR1A1, 2	Thyroid hormone (T3)	H
Retinoic acid receptor	RARα, β, γ	NR1B1, 2, 3	Retinoic acid	H
Peroxisome proliferator activated receptor	PPARα, β, γ	NR1C1, 2, 3	Benzotriene B4; Wy 14.643 Eicosanoids; thiazolidinediones (TZDS); 15-deoxy-12,41-prostaglandin J2; polyunsaturated fatty acids	H
Reverse ErbA	RevErbα, β	NR1D1, 2	Unknown	M, D
RAR-related orphan receptor	RORα, β, γ	NR1F1, 2, 3	Cholesterol, cholesterol sulphate, retinoic acid	H
Liver X receptor	LXRα, β	NR1H3, 2	Oxysterols	H
Farnesoid X receptor	FXRα, β	NR1H4, 5	Bile acids	H
Vitamin D receptor	VDR	NR1I1	1-25$(OH)_2$ vitamin D_3	H
Pregnane X receptor	PXR	NR1I2	Xenobiotics, pregnanes; C21 steroids	H
Constitutive androstane receptor	CAR	NR1I3	Xenobiotics, phenobarbital	H
Hepatocyte nuclear factor 4	HNF4α, γ	NR2A1, 2	Fatty acyl CoA thioesters	D
Retinoid X receptor	RXRα, β, γ	NR2B1,2,3	9-*Cis*-retinoic acid	D
Chicken ovalbumin upstream promoter transcription factor	COUP-TFI, II	NR2F1, 2	Unknown	D, H
Estrogen receptor	ERα, β	NR3A1, 2	Estradiol	D
ER-related receptor	ERRα, β, γ	NR3B1, 2, 3	Unknown, diethylstilbestrol 4-OH tamoxifen	M, D
Glucocorticoid receptor	GR	NR3C1	Glucocorticoids	D
Mineralocorticoid receptor	MR	NR3C2	Aldosterone, spirolactone	D
Progesterone receptor	PR	NR3C3	Progestins	D
Androgen receptor	AR	NR3C4	Androgens	D
NGF-induced factor B	NGFI-B	NR4A1	Unknown	M, D, H
Steroidogenic factor 1	SF-1	NR5A1	Unknown	M
Germ cell nuclear factor	GCNF	NR6A1	Unknown	D
Dosage sensitive sex reversal	DAX-1	NR0B1	Unknown	

M: Monomer, D: Dimer, H: Heterodimer

NRs regulate transcription of target genes by binding to specific DNA sequences known as hormone response elements (HREs) located in regulatory regions (promoters or enhancers) of the gene. NRs are initially activated through different mechanisms depending on their subclasses. The subfamily III steroid receptors, with the exception of ERs, recognise a HRE which is characterized by a palindrome/inverted repeat of a core hexanucleotide consensus sequence (AGAACA) spaced by three nucleotides. ERs bind to the consensus AGGTCA motifs with the same configuration. In the absence of hormones, steroid receptors exist as inactive protein complexes bound to heat shock proteins (Hsps), which help to maintain the receptors in a functionally folded state and at the same time to keep the receptors off from the DNA. Steroid hormones can enter their target cells by simple or facilitated diffusion through cell membranes. Upon binding to hormones/ligands, steroid receptors undergo conformational changes, dissociate from the Hsps, dimerize, bind to target HREs and subsequently activate transcription of the target genes (Figure 2A). Many receptors for non-steroidal hormones such as TRs, RARs, PPARs, and VDRs (subfamily I) appear to bind tightly to HREs constitutively, predominantly as a heterodimer with RXR, and confer an active transcriptional repression on their target genes. Therefore, activation of these receptors occurs primarily as steps subsequent to DNA-binding and ligand-binding which also lead to changes in their conformation (Fig 2B). The configuration of the non-steroidal receptor HREs may be palindromes, direct repeats or inverted palindromes of the core hexanucleotide AGGTCA with variable nucleotide spacing in between (reviewed in (6)). Many of the orphan receptors may still have unidentified ligands, while others may be activated constitutively or by other means than ligands. Orphan NRs are capable of binding to direct repeat HREs as heterodimers with RXR or homodimers, as well as recognising extended half sites HREs (AGG/TTCA) as monomers (reviewed in (7)). Despite the difference in their modes of initial activation, all NRs require association of transcriptional coregulator proteins for subsequent control of transcription activation of their target genes (see section 2).

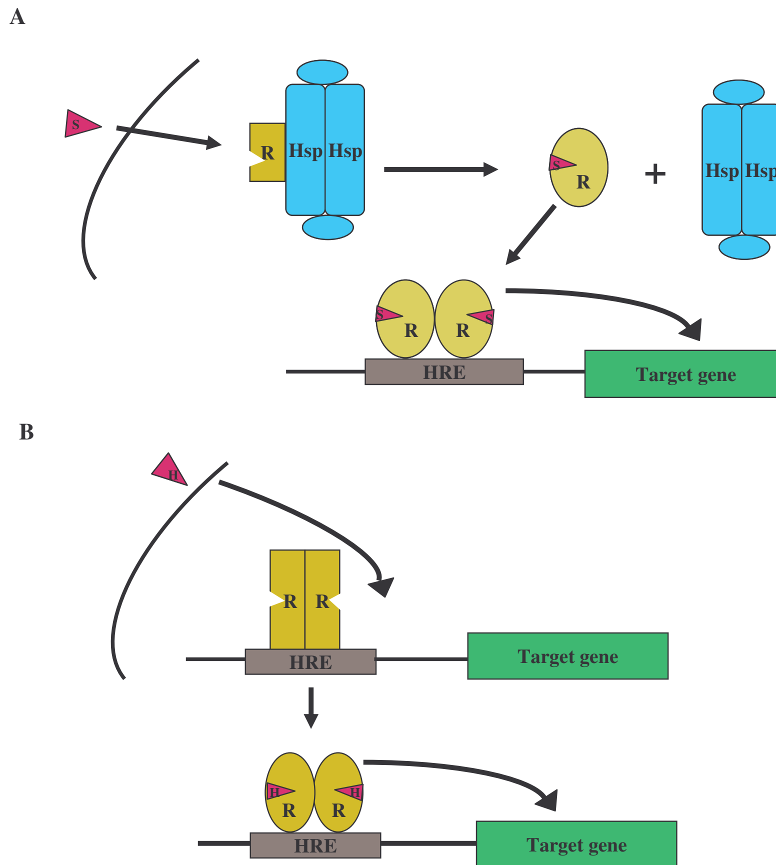

Figure 2. Mechanisms for activation of steroid and non-steroidal receptors (edited from (8)). A, Steroid receptors (R) associate with chaperon heat shock proteins (Hsp) and are in inactive state in the absence ligands. Upon ligand binding, the receptors change their conformation, dissociate from the chaperon complex, dimerize and bind to their hormone response element (HRE) on target genes. Transcription of the target gene is subsequently activated. B, Certain non-steroid receptors (R) such as TRs, PPARs, and RARs are intrinsically bound to their HREs as homodimer or heterodimer with RXRs in the absence of ligands, and repress transcription of target gene. Ligand binding changes the receptor conformation, releases its repressive function, and activates formation of transcription complex to initiate transcription.

1.3. Steroidogenic factor-1

1.3.1. Functional and structural domains

The orphan nuclear receptor Steroidogenic factor 1 (SF-1, also designated as Ad4BP/NR5A1) plays central roles in endocrine development and function at multiple levels of the hypothalamic-pituitary-steroidogenic axis (reviewed in (9)). The protein binds to a shared AGGTCA (Ad4) motif in the promoter region of genes encoding cytochrome P450 steroid hydroxylases of steroidogenesis, and is a key mediator for the tissue-specific expression of these genes (10,11). SF-1 was first recognised as a member of the NR superfamily through its high homology to the Drosophila nuclear receptor fushi tarazu factor 1 (12,13). Like other NR family members, SF-1 contains all the common functional and structural domains for a NR, i.e a characteristic zinc finger DBD, an intervening hinge region and a LBD which harbors an

AF-2. SF-1 does not possess a classical AF-1, but has two other activating domains downstream to the DBD named the proximal activation function (pAF) harboring the serine residue at 203 (also called AF-1), and the FP region, which both cooperate with the AF-2 and are required for maximal activity of SF-1 (reviewed in (14)) (Fig 1B). The AF-2 of SF-1 harbors a LLIEML conserved sequence which is necessary for transactivation of SF-1 target genes (15-17). This domain of SF-1 has been implicated in the transduction of the cAMP signal to transcriptional regulation of SF-1 target genes (18), and in interaction with several coactivator proteins (reviewed in (9,14)). SF-1 binds to its response DNA element as a monomer (14).

1.3.2. Expression profile and biological roles

SF-1 is specifically expressed in steroidogenic tissues including the adrenal cortex, ovarian theca and granulosa cells, as well as testicular Leydig cells (12,13). Studies on the expression profile of SF-1 during mouse embryonic development have revealed that SF-1 is expressed in the steroidogenic tissues from the earliest stages of organogenesis (19). In the later stages of embryonic development, SF-1 is expressed persistently in testes but diminish in ovaries (19,20). SF-1 is also expressed in the developing pituitary gland and in the precursor to the endocrine hypothalamus (diencephalons) (19).

The essential roles of SF-1 in endocrine development and function have been clearly demonstrated by the development of targeted gene disruption in embryonic stem cells to create mice that are globally or tissue-specifically lacking SF-1. SF-1-knockout mice did not develop gonads or adrenal glands, died shortly after birth due to adrenal insufficiency, and exhibited a male-to-female sex reversal of their internal and external genitalia (21,22). The expression of several markers of pituitary gonadotrope cells including luteinizing hormone (LH) β, follicle-stimulating hormone (FSH) β, the α-subunit of glycoprotein hormones and the receptor for gonadotropin releasing hormone, was also impaired in SF-1 knockout mice (23,24). Furthermore, knockout mice showed agenesis of the ventromedial hypothalamic nucleus which is linked to feeding and appetite regulation and female reproductive behavior (24-26), and showed defects in spleen development (27). Pituitary-specific ablation of SF-1 in mice has confirmed the pituitary origin of the absence of gonadotropin markers and delineated further that it is the absence of the gonadotropins that causes gonadal defects (26).

In humans, function-disrupting mutations and polymorphisms of SF-1 protein have been found to be associated with certain defects and disorders of adrenocortical development and/or sexual differentiation. One group has found a heterozygous G35E mutation that

caused XY reversal and adrenal failure in the patient (28). The mutation occurred at the proximal box of the first zinc finger that abolishes DNA binding ability of SF-1. Another group has identified a R255L heterozygous mutation in a 46 XX karyotype patient with normal ovary but with adrenal insufficiency (29). A homozygous R92Q mutation that leads to partial loss in the DNA-binding ability of SF-1 was also isolated in an infant patient with adrenal failure and complete 46XY sex reversal (30). Furthermore, in XY sex-reversal patients with gonadal agenesis, but with normal adrenal function, two types of SF-1 mutations were isolated, either a 8bp microdeletion that leads to early termination upstream of the AF-2 domain or a C16X heterozygous mutation causing premature termination and abolishment of SF-1 activity (31,32). Gly146Ala polymorphism in the SF-1 sequence has also been identified and implicated as a susceptible factor for development of severe micropenis (33), development of type 2 diabetes and impaired insulin action in Chinese (34).

1.3.3. Transcriptional regulation of target genes

Accordant to its important roles in endocrine organogenesis and function, SF-1 regulates the expression of many genes involved in the endocrine processes along the hypothalamic-pituitary-gonadal axis, as summarized in Table 2 (reviewed in (9,14)). Moreover, many of the SF-1 target genes encode major enzymes of the adrenal steroid biosynthesis, such as the cytochrome P450 cholesterol side-chain cleavage enzyme, 17α-hydroxylase (CYP17), 3β-hydroxysteroid dehydrogenase, 21-hydroxylase (CYP21), and the steroidogenic acute regulatory protein (StAR). In most of the cases, SF-1 activates the basal expression of its target gene, and the transcriptional activation is induced constitutively in the absence of any exogenous ligands. It is still unclear whether the transcriptional activity of SF-1 is regulated by a physiological ligand in these cases. Although SF-1 was reported to be activated by oxysterols in certain cellular contexts (35), these molecules seem not to be its *bona fide* ligand (36). Recently, two independent groups have revealed the crystal structure of SF-1 and demonstrated that the second messenger phosphatidyl inositol (phospholipid) binds to SF-1 in its ligand binding pocket in the LBD, and that the ligand binding is required for maximal activity of SF-1 (37,38). However, the contribution of a ligand in regulation of SF-1 target genes remains to be elucidated.

Table 2. SF-1 target genes. SF-1 activates the basal activity of most of the genes except the gene encoding LH receptor in gonads/leydig cells whose basal transcription is inhibited. Transcriptional induction or inhibition of the genes by activation of the cAMP/PKA pathway is also indicated (9,14).

Site of action	Target gene	Basal activity/ Induction
Adrenal cortex	Gonadotropin-releasing hormone receptor	Basal
	Cytochrome P450 steroid hydroxylases	basal + induction by cAMP (except for CYP11B2)
	3ß-hydroxysteroid dehydrogenase	basal
	Steroidogenic acute regulatory protein (StAR)	basal + induction by cAMP
	Corticotropin receptor	basal + induction by cAMP
	Scavenger receptor-B1	basal + induction by cAMP
	Hydromethylglutaryl-CoA reductase	basal
	DAX-1	basal
	Aldose reductase-like protein	basal
Gonads		
Leydig cells	Cytochrome P450 steroid hydroxylases	basal + induction by cAMP
	StAR	basal + induction by cAMP
	LH receptor	basal inhibition
	Insulin-like polypeptide 3	basal
	Prolactin receptor	basal
	Mullerian inhibiting substance (MIS) receptor	basal
Sertoli cells	MIS	basal
	Inhibin	basal + induction by cAMP
	FSH receptor	basal + inhibition by PKA
	Sex-determining region Y (SRY)	basal + inhibition by cAMP
	SOX9 (SRY box)	
Theca and granulosa cells	Cytochrome P450 steroid hydroxylases	basal + induction by cAMP
	StAR	basal + induction by cAMP
	Inhibin	basal + induction by cAMP
	Oxytocin	basal
Pituitary		
Gonadotropes	α-subunit of glycoprotein hormones	basal + induction by GnRH
	Luteinizing hormone (LH) β	basal + induction by GnRH
	Follicle-stimulating hormone (FSH) β	basal
	Gonadotropin-releasing hormone receptor	basal
Ventromedial hypothalamic nucleus	N-methyl-D-aspartate receptor	

The absence of an exogenous ligand in activation of SF-1 has raised speculation on whether the receptor is regulated by alternative mechanisms involving signal transduction pathways. Considerable attention has been focused on the regulation of SF-1-mediated transcriptional activation in response to physiological cues, especially those involved in the endocrine homeostasis. cAMP is the intracellular secondary messenger molecule that transmits the signal of several extracellular tropic hormones, including the adrenocorticotropic hormone (ACTH) to cAMP-dependent protein kinase A (PKA) and the downstream signaling pathway (described in 3.2) (reviewed in (39,40)). Activation of the cAMP/PKA signaling

pathway has been shown to enhance SF-1-dependent gene transcription (41) and SF-1 seems to be required for cAMP-dependent expression of several genes encoding steroidogenic enzymes (42-46). While it has been demonstrated *in vivo* that SF-1 mRNA accumulation in the adrenals is not affected by an increased ACTH level (47), the effect of cAMP/PKA pathway activation on SF-1 expression appears to be far more complex and controversial in cell culture systems. The expression level of SF-1 can be unaffected or increased depending on cell types that are subjected to study (14). There is also evidence showing that activation of the PKA signaling pathway leads to an accumulation of SF-1 protein due to increased protein stability (48). One model that is recently developed from efforts to elucidate the molecular mechanism underlying regulation of SF-1 transcriptional activity by endocrine signals involves posttranslational modifications of the receptor and the implication of coregulators. Although it was demonstrated that SF-1 is phosphorylated *in vitro* by PKA (49), mutation of the serine residue in the consensus motif for PKA phosphorylation did not affect SF-1 function in transfection assays (50). In addition, reports have suggested that the protein is not phosphorylated *in vivo* by PKA (48,51). However, Hammer and coworkers (51) have reported that the mitogen activated protein kinase (MAPK) phosphorylates SF-1 at a serine residue (Ser203) which is located in the AF-1 domain, and this phosphorylation leads to an enhanced transcriptional activation of SF-1 target genes and alterations in recruitment of selective nuclear cofactors. SF-1 is also subjected to sumoylation which represses its activity through stimulating recruitment of transcriptional repressors and/or relocation of SF-1 to nuclear speckles (52-54). Acetylation has also been observed as another mode of posttranslational modification of SF-1 that potentiates its transcriptional activity and mediates recruitment of coactivator proteins (55,56). Intriguingly, a recent study has reported that activation of the cAMP pathway increases SF-1 acetylation (56). Furthermore, Winnay and Hammer (57) have very recently demonstrated that the ACTH-dependent-signaling pathway modulates the temporal dynamic assembly of SF-1 and its associated coactivators on the promoter of a SF-1 target gene encoding the melanocortin 2 receptor. The role of coregulator proteins in the transcriptional regulation by SF-1 and other NRs is further described in the following sections.

2. Nuclear receptor coregulators

DNA/HRE-binding or target gene recognition, which is either ligand-activated or constitutive, is generally a first essential step in a process of transcriptional regulation by a NR. However, subsequent recruitments of transcriptional cofactors/coregulators to the HRE-bound NR

determine the magnitude of transcriptional activity, either in an enhancing or a repressive manner, depending on the type of recruited transcriptional coregulators (classified as coactivators and corepressors). The concept for the existence of limiting transcriptional cofactors other than RNA polymerase and the basal transcription machinery, which are necessary for gene expression, came from the observation of transcriptional quenching/interference among different receptors (58,59). Later studies employing biochemical strategies provided the first evidence that ER associated directly with at least three mammalian proteins of 160, 140 and 80 kDa in a ligand- and LDB-dependent manner (60,61). The proteins were speculated to modulate the ligand-dependent transcriptional activation by ER. Following this finding, several coregulator proteins known as coactivators or corepressors have been cloned and characterized (reviewed in (6,62-65)). Coregulators are diverse both structurally and in the way they participate in the NR-dependent transcription process through their different enzymatic activities. Table 3 summarizes recently characterized properties and coregulatory roles of selected transcription coactivators and corepressors in the transcriptional regulation by NRs.

2.1. Coregulator classes and regulatory roles in NR-dependent transcription

Coactivators and corepressors do not bind to DNA, but associate with the DNA-bound NR in a sequential, combinatorial and temporally-regulated manner (64). Once recruited to HRE-bound NRs, coactivator protein complexes stimulate the transcriptional activity by participating in chromatin remodeling and acting as bridging molecules for the recruitment and modification of RNA polymerase and the basal transcription machinery, including the template-activating factors and TATA-binding protein (TBP) (64). Five major classes of coactivators have been identified: i) coactivators of the p160/steroid receptor coactivator (SRC) family (described in section 2.3), ii) histone acetyltransferases (HATs)-possessing coactivators including CBP/p300 and pCAF/GCN5 which modulate chromatin structure and other transcriptional factors through acetylation (reviewed in (67)), iii) histone arginine methyltransferases (HMTs) such as CARM1 and PRMT1 which are able to methylate histones as well as other proteins (reviewed in (68)), iv) ATP-dependent nucleosome remodeling complexes such as SWI/SNF (reviewed in (69,70)), and v) the multisubunit mediator complex TRAP/DRIP/ARC which participate in transcription process by bridging the basal transcription machinery to the promoter-bound NR-coregulator complex (reviewed in (71,72)) (Table 3). Recruitment of these coactivator complexes to DNA-bound NRs and their coactivator actions in transcriptional activation is described further in section 2.3.2.

Table 3. Nuclear receptor coactivators and corepressors. Properties and roles of selected coactivators and corepressors in transcriptional regulation by NRs are briefly specified (6,62-66).

Coregulators	Denomination	Properties	Role
SRC-1 SRC-2/GRIP1/TIF2 SRC-3/p/CIP/ AIB1/ACTR/ RAC3/TRAM-1	Steroid receptor coactivator-1 Steroid receptor coactivator-2/ GR-interacting protein 1/ Transcription intermediary factor-2 Steroid receptor coactivator-2/ p300/CBP/cointegrator-associated protein/Amplified in breast cancer 1/ Activator of the thyroid and retinoic acid receptor/Receptor-associated coactivator 3/TR activator molecule 1	Histone acetyltransferases (HAT), signal integrating	Coactivators (see section 2.3)
p300 CBP p/CAF	300-kD protein cAMP-response element binding (CREB)-binding protein p300/CBP-associated protein	Acetyltransferases for histones and other proteins	Coactivators
CARM1 PRMT1	Coactivator-associated arginine methyltransferase 1 Protein arginine methyltransferase 1	Histone and CBP methyltransferases	Coactivators
BRG-1/hBrm (SWI/SNF)	Brahma (fly)-related gene 1/ Human homolog of BRG	ATP-dependent chromatin remodeling	Coactivator
TRAP/DRIP/ARC	TR-associated protein/VDR-interacting protein/Activator-recruited cofactor	Reinitiation/maintenance complex-assembling	Coactivators
PGC-1	PPARγ coactivator-1	Splicing-controlling, signal integrating	Coactivator
CoAA	Coactivator activator	Splicing-controlling	Coactivator
E6-AP	E6-associated protein	Ubiquitin ligase	Coactivator
UbcH7	Ubiquitin-conjugating enzyme 7	Ubiquitin-conjugating	Coactivator
TBP-1	TATA-binding protein-1	Initiation complex-assembling, ATPase	Basal transcription machinery
PNRC/PNRC2	Proline-rich nuclear receptor co-regulatory protein		Coactivators
GCN5	General control nonderepressed 5	HAT	Coactivator
RIP140	Receptor-interacting protein 140	Signal-integrating	Coactivator or corepressor (see section 2.4)
LCoR	Ligand-dependent corepressor	Signal-integrating	Coactivator/ corepressor
SMRT/NCoR	Silencing mediator of retinoic acid and thyroid hormone receptor/ Nuclear receptor corepressor	Chromatin remodeling HDAC-recruiting	Corepressors
mSin3/HDACs	Human homolog of yeast corepressor Sin3/Histone deacetylases	Histone deacetylation, ATP-dependent chromatin remodeling	Corepressors
SUN-CoR	Small ubiquitous nuclear corepressor	16-kDa nuclear protein with intrinsic repression domain	Corepressor

Corepressor proteins, which are exemplified by NR corepressor (N-CoR) and the silencing mediator of retinoid and thyroid receptors (SMRT), play comparable important roles by negatively regulating the NR-mediated gene expression (reviewed in (73,74)). N-CoR and SMRT are constitutively associated with certain unliganded NRs, particularly NRs of the subfamily I such as TR, RARs and VDR, enabling the receptors to actively repress the basal transcription in the absence of their cognate ligands (reviewed in (74)). Upon hormone-binding, corepressor proteins dissociate from receptors and allow the receptors to bind to coactivators and activate gene expression of target genes (75). The corepressors do not appear to interact with steroid hormone receptors in the absence of ligands. However, they are recruited to steroid receptors in the presence of receptor antagonists and mediate release of coactivators and transcription repression on receptor target genes (64). This suggests a possible existence of a cellular equilibrium of coactivators and corepressors that can be favored toward the corepressors in the presence of antagonists, and that has been implicated as a mechanism for the antagonist-associated effects of steroid hormone receptors (64). N-CoR and SMRT have also been demonstrated to confer transcriptional repression on several other members of the NR superfamily, including v-ErbA, RevErb, PPARα, and DAX-1 (Dosage sensitive sex reversal) (reviewed in (76)).

Coregulator proteins that bind to NRs in a ligand/agonist-dependent manner and have mixed coactivator-corepressor functions have also been identified. These include receptor-interacting protein 140 (RIP140) (77-81) and a recently identified ligand-dependent corepressor (LCoR) (82). It has been suggested that these coregulator proteins are involved in hormone-dependent repression and in attenuation of agonist-induced transactivation of target genes by NRs (66). A brief description of RIP140 and its function in repression of NR-dependent gene expression is presented in section 2.4. Other proteins that have been associated with negative regulation of NR transcriptional activity are the transcriptional intermediary protein 1α, nuclear receptor-binding SET domain containing protein and the small ubiquitous nuclear corepressor (SUN-CoR). SUN-CoR has no homology to N-CoR or SMRT and is thought to function as an additional component of the corepressor complex involved in transcriptional repression of unliganded NRs (83).

Corepressor proteins seem not to possess intrinsic repressive activity. Accumulated data has suggested that they associate with different classes of histone deacetylase (HDAC) factors, becoming part of larger multiprotein complexes which are able to modulate chromatin via histone deacetylation and counteract gene activation mediated by HAT-containing coactivator complexes (reviewed in (6,63,66,76)). Furthermore, it has been demonstrated that

corepressor proteins interact with and inhibit the activity of basal transcription factors (84,85). It is also proposed that corepressor binding leads to a masking of the transactivation domain of coactivators or inhibition of NR-coactivator interaction (74).

2.2. Structural determinants for the interaction with NRs

Coactivators have been observed to interact with NRs both at the receptor AF-1 and AF-2 domain and contribute to modulation of NR-dependent transcriptional activation (reviewed in (3)). For example, the transcriptional coactivators TBP, the cAMP response element binding protein-binding protein (CBP) and the vitamin D receptor-interacting protein 150 can interact with the AF-1 region of GR (86-88). TBP has been also reported to associate with the N-terminal domain of ERs (89). Most of the AF-1-associated coactivators contain no conserved sequence or structural homology, and the structural basis for AF-1-coactivator interactions is not well understood. It has been proposed that binding of AF-1 coactivators leads to conformational changes at the AF-1 surface (3). Coactivators that have been identified to interact with NR at the AF-2 domain (also called AF-2 coactivators) contain conserved leucine-rich motifs characterized by the consensus sequence LXXLL (where L is leucine and X is any amino acid) and display interaction with the NRs in a ligand-dependent manner (78). The LXXLL motif has been shown to be essential and sufficient for the interaction with NRs and multiple LXXLL motifs are usually found within a NR-interacting domain of coactivators (78,90). Studies on crystal structures of ligand-bound LBD/LXXLL-containing peptide complex have suggested that changes in conformation of a NR upon ligand/agonist binding create an optimal binding surface for the LXXLL motifs of a coactivator (91-93).

Similar to coactivators, corepressor proteins contain helical leucine-rich LXXLL motifs or LXXLL-related motifs named CoRNR boxes that are required for their interaction with NRs (reviewed in (66,76). However, corepressors require different structural determinants on NRs for interaction, depending on which subfamily the receptors belong to. In many cases of subfamily I NRs, the AF-2 domain is not required for binding to corepressor proteins, but rather serves to trigger the dissociation of corepressors from the receptors (6). A domain named CoR box which encompasses the hinge region and helix 1 of the LBD of TR and RAR is found to be essential for interaction of the receptors with N-CoR (94,95). This interaction domain is also noted to be relatively conserved among TR, v-ErbA and RAR, but not among other NRs that do not naturally associate with the corepressor. In other cases, the AF-2 helix of NRs has been suggested as an important regulator/determinant of coactivator-corepressor recruitment exchanges in responses to antagonist-binding, as characterized for PPAR (96).

Co-crystallization of antagonist-bound PPAR has demonstrated that in the presence of antagonists, the AF-2 domain of PPAR is oriented in a way that forms a binding pocket favorable for the binding of corepressor over coactivators. However, mapping of the interaction domain of steroid receptors with corepressors has revealed that the receptor amino-terminal regions are essential for corepressor interaction (97,98).

2.3. The p160/Steroid receptor coactivator family

2.3.1. Members and functional domains

Members of the p160/steroid receptor coactivator (SRC) family include (i) steroid receptor coactivator 1 (SRC-1) or nuclear receptor coactivator-1 (NCoA-1) (99), (ii) transcription intermediary factor 2 (TIF2) and the mouse homolog glucocorticoid receptor-interacting protein (GRIP1) (also termed SRC-2 or NCoA-2) (100,101), and (iii) p300/CBP cointegrator protein (p/CIP) (102) and its human homologues: amplified in breast cancer-1 (AIB1) (103), activator of the thyroid and retinoic acid receptor (ACTR) (104), thyroid hormone receptor activator molecule (TRAM 1) (105), and receptor-associated coactivator 3 (RAC3) (106) (also termed SRC-3 or NCoA-3). The coactivators are genetically distinct but structurally and functionally related. They are able to interact with and enhance transcriptional activity of multiple NRs ranging from steroid hormone receptors (ER, GR, PR) to non-steroidal hormone receptors (TR, RXR, RAR, PPARγ) as well as orphan nuclear receptors (HNF-4) in a ligand-dependent manner (107).

The p160/SRC members are about 160 kDa in size and share an overall sequence similarity of 50-55% and sequence identity of 43-48% (108). The general structure of a p160/SRC member has been characterized to contain a highly conserved N-terminal basic helix-loop-helix (bHLH)/Per/ARNT/Sim domain (bHLH/PAS), a relatively conserved central region (the NR-interacting domain) containing three LXXLL motifs or NR boxes (L1-L3) that are responsible for interaction with ligand-bound NRs, and two intrinsic transcriptional activation domains (AD1 and AD2) (Figure 3) (107). The AD1 is involved in the interaction with HAT-possessing coactivators (CBP/p300). In addition, this domain contains other three LXXLL-like motifs (L4-L6) which are required for CBP/p300 interaction and the coactivation function of the p160/SRC proteins (102,109,110). AD2 contributes to coactivation of NR-dependent transcription by participating in the interaction with histone methyltransferases (HMT) CARM1 (coactivator-associated arginine methyltransferase 1) and PRMT1 (protein arginine methyltransferase 1) (111,112).

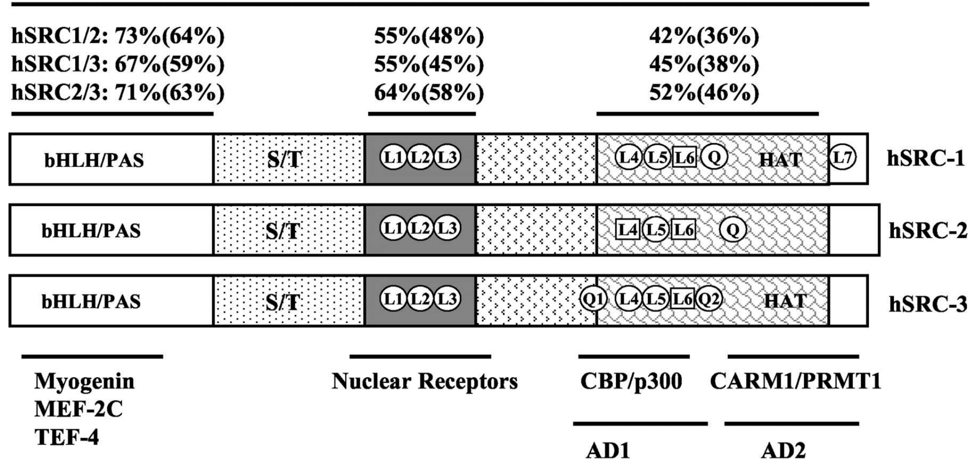

Figure 3. Structural and functional domains of SRC-1, SRC-2 and SRC-3 (adapted from (108)). Members of the p160/SRC family share a common structure containing a basic helix-loop-helix/Per-ARNT-Sim homologous (bHLH/PAS) domain, a serine/threonine (S/T) rich region, a NR-interacting domain with three LXXLL (encircled L) motifs, and two activation domains AD1 and AD2 which are located to the C-terminal region, encompass glutamine-rich sequences (Q) and several other typical or atypical (boxed L) LXXLL motifs. The sequence similarity between the p160/SRC members and selected proteins that interact with different domains of the coactivators are indicated above and below the bars respectively.

A third activation domain (AD3) which is located in the bHLH/PAS domain of GRIP1 has been identified. This AD3 enhances transcriptional activation by ER and other NRs through recruiting a secondary coactivator known as coiled-coil activator (CoCoA) (113). Moreover some parts in the C-terminal region of GRIP1 may regulate its own AD1- and AD2-dependent coactivator activity through acting as self-association or repression domains (114). The amino acid sequence 1350-1400, named the self-association domain, was thought to mediate intramolecular and intermolecular interactions of GRIP1, leading to changes in the protein conformation which are favourable or unfavourable for transactivation and coactivation activity. The repression domains defined by several sequences of the C-terminal region were proposed to modulate the effects of associated corepressor proteins (114). GRIP1 also contains an intrinsic corepression domain which is mapped within the amino acid sequence 765-1007 and responsible for GR-mediated repression at the GR response element tethered to the activator protein 1 or the nuclear factor-kappaB (NF-kappaB) (115).

2.3.2. Mechanisms of coactivator action

The carboxyl-terminus of SRC-1 and ACTR/SRC-3 possesses intrinsic HAT properties (104,116), which may also be directly involved in chromatin remodeling. However, it is believed that p160 family coactivators play central roles in the potentiation of NR-dependent transcription by mainly serving as adapters for direct or indirect recruitment of other chromatin-remodeling coactivator complexes, including CBP/p300/pCAF (p300/CBP-associated factor), CARM1/PRMT1, and SWI/SNF (107). Based on accumulated data, a general model for the sequential molecular mechanism of p160/SRCs function has been proposed (64,117), as illustrated in Figure 4. Briefly, p160/SRC proteins existing in complexes with CBP, p300, p/CAF, CARM1 and PRMT1 are first recruited to a target promoter through their direct interaction with ligand-activated and promoter-bound NRs. This leads primarily to acetylation and methylation of specific histones. The SWI/SNF (Brg/Brm) complex is then recruited to promoter-bound NRs through direct or indirect associations with CBP/p300, which results in ATP-dependent acetylation of specific histones and subsequent changes in local chromatin structure. Recruitment of the TRAP/DRIP complex can occur thereafter through interactions with the SRC/CBP/p300 complex or direct interactions with NRs. This complex associates directly with the basal transcription machinery and therefore initiates transcription. Studies employing chromatin immunoprecipitation (ChiP) assays to characterize recruitment of several coregulator proteins by ER to its natural target promoters have supported the roles of p160/SRCs as docking or scaffold proteins for the assembly of other coactivator complexes at promoter-bound NRs (118-120). These studies have also suggested that recruitments of coactivator complexes occur dynamically and in a cyclic manner. In addition, the presence of ubiquitin ligases such as E6-associated protein (AP) and MDM2 and the 26S proteasome components, which are responsible for specific protein degradation (see section 3.4), have been observed in the protein complexes recruited to the ER target promoter, suggesting a subsequent downstream proteasome-dependent turnover of the receptor-coactivator complex in the transcription activation process (118-120).

The coactivators of the p160/SRC family are in fact recruited preferentially and differentially to promoter-bound NRs in different cellular contexts including promoters, ligands and cell types. For example, SRC-1 and SRC-2 exhibit similar, but not identical preferences for binding to nine different NRs, of which AR interacts well with SRC-2 but poorly to SRC-1 (121). In addition, ERβ recruits AIB1 and TIF2 differentially to different estrogen receptor responsive elements (122). While binding of PPARγ to the ligand F-L-Leu induces a permissive association with SRC-1 (and p300), binding of the receptor to

rosiglitazone preferentially resulted in TIF2 recruitment (123). Moreover, the ligand-induced transcriptional activity of PR is modulated primarily by SRC-3 in the luminal epithelium of the mammary gland, but primarily by SRC-1 in the stroma compartment of the uterus (124). In a recent study where four different thyroid hormone-regulated target genes were examined, it has been observed that not only each gene had a different recruitment preference for SRC-1 and GRIP1, but also the temporal recruitment patterns of SRC-1 and GRIP1 were different from gene to gene (125).

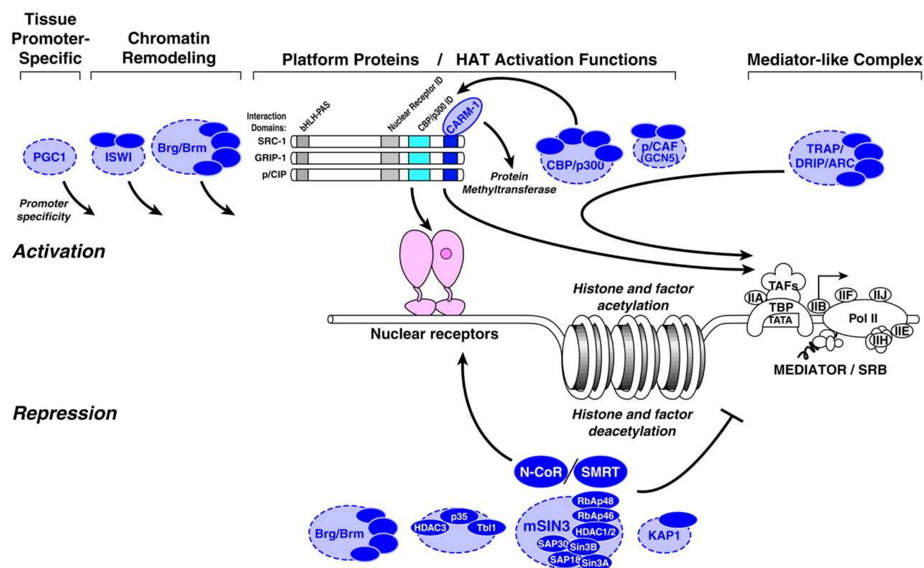

Figure 4. A hypothetical model for the exchange and sequential recruitment of coactivators and corepressors in regulation of NR-dependent gene expression (adapted from (117)). In an activated state due to ligand binding or other cellular signals, promoter-bound NRs recruit coactivators of the p160/SRC family which essentially serve as a scaffold to complex with other coactivator proteins including the histone acetylase CBP/p300 and p/CAF(GCN5), and the methylase CARM1/PRMT. The coactivator complexes are involved in chromatin remodeling. Following this step is recruitment of the ATP-dependent chromatin remodeler complex SWI/SNF (Brg/Brm) and the transcription mediator complex TRAP/DRIP/ARC which make direct contacts with the basal transcription machinery to initiate transcription. Association of NRs with the corepressor complexes including N-CoR/SMRT leads to transcriptional repression.

The molecular basis of NR preferences for coactivators has been linked to the structure of the NR with focus on its LBD (125-128) and the NR boxes of coactivators (91,92,121,129-132). Specific promoter- and/or ligand-binding induces changes in NR structure which provide interaction surfaces permissive to certain coactivators but not others. In addition, coactivators require different LXXLL-motifs in their interaction with NRs. For instance, although NR boxes 2 and 3 display crucial roles for binding of GRIP1 to most NRs, mutations in each of these NR boxes differently affected the interactions of GRIP1 with ER and AR or GR (121,130). Additionally, LBDs of GR and TR exhibit different affinities for these two NR

boxes (91). It is believed that the preferential and differential recruitment of the p160/SRCs by specific NRs determines the subsequent assembly of specific downstream coactivator complexes on target genes. This has been in part supported by the observation that the p160/SRC family members exhibit differential affinities for CBP, whereby CBP interacts preferentially with SRC-3 over SRC-1 and SRC-2 (102). Furthermore, it has been demonstrated that upon ligand binding PR interacts preferentially with SRC-1 which leads to recruitment of CBP and an enhanced acetylation of histone H4. In contrast, GR prefers interaction with SRC-2 and subsequently recruit pCAF which results in enhancements of specific acetylation, hypomethylation and phosphorylation of histone H3 (128).

2.3.3. Expression profile and biological roles

The members of the p160/SRC family are widely expressed and have been detected in several tissues including placenta, testis, pancreas, lung, kidney, liver and brain. However, the relative expression level of each coactivator member as well as among the coactivators varies between cell types and tissues (reviewed in (107,108)). For example, mouse SRC-3 is predominantly expressed in epithelial cells of mammary gland, oocytes, vaginal epithelial layer, hepatocytes and in the smooth muscle cells of blood vessels, intestines and oviducts (133,134). SRC-1 is expressed at a higher level than SRC-2 and SRC-3 in the cerebella Purkinje cells (PCs) (135). While the overlapping distribution of the p160/SRC members in various tissues implies possible functional redundancies between the coactivators, their variable tissue-specific expression suggests that each member of the family may exhibit differential and preferential biological functions in certain cell types/tissues with certain receptors.

The biological importance of the p160/SRC family members have been partially characterized by gene-ablation studies performed in mice, as reviewed in ref (108). Specific phenotypes observed in mice lacking either of the genes encoding the coactivators are summarized in Table 4. Particularly, SRC-1 is implicated in mediating actions of steroid hormones via the transcriptional activity of steroid hormone receptors (136,137) and is required for thyroid hormone-dependent downregulation of thyroid stimulating hormones (138). SRC-1 plays also pivotal roles in brain development and function, as well as in regulation of energy metabolism (135,139). SRC-2/GRIP1 has major roles in reproduction, lipid metabolism and energy balance (139,140). However, SRC-1 and GRIP1 seems to be differently involved in the regulation of energy homeostasis, as evidenced by different phenotypic outcomes of SRC-1 and SRC-2 knock out mice (Table 4). Interestingly, the expression level of GRIP1 in both brown (BAT) and white (WAT) adipose tissues, as well as

the expression ratio between SRC-1 and GRIP1 is enhanced in mice that are subjected to a high fat diet (139). The recent identification of significant downregulation in gene expressions of several key regulatory enzymes of energy metabolism upon ablation of GRIP1 gene has strengthened the pivotal roles of GRIP1 in energy homeostasis (141). Unlike the other two members, SRC-3 is critically important for cellular growth and female reproductive function and development (133,142). SRC-3 is also essential for vascular cell proliferation and inflammatory and immune responses (134,143). Notably, SRC-3 mRNA is expressed at extremely high levels in ER-positive breast and ovarian tumors and cancer cell lines (reviewed in (108)), suggesting a potential role of SRC-3 in development of breast carcinogenesis. Altogether, the observations have indicated that the p160/SRC members possess a functional specificity and play important roles in different biological processes, especially reproduction and metabolism.

Table 4. The Major Phenotypes Observed in p160/SRC Knockout (KO) Mice.

KO mice	Phenotypes	References
SRC-1	Normal growth and fertility; partial steroid and thyroid resistance; delayed development of cerebellar PC; moderate motor learning dysfunction; no effect on PPARγ-regulated genes in liver; impaired thermogenesis and decreased energy expenditure in brown fat	(135-139)
SRC-2	Normal somatic growth; defective spermatogenesis; testicular degeneration; placental hypoplasia; reduced fertility in both male and female; lower fat accumulation and higher lipolysis in white adipose tissue; higher energy expenditure in brown adipose tissue; resistance to obesity	(139-141)
SRC-3	Somatic growth retardation; delayed growth of puberty and mammary gland; lower levels of IGF-1 and estrogen; reduced female fertility; reduced ER-dependent vascular protection; impaired expression of interferon-regulatory factor 1 by NF-kappa B	(133,134,142,143)

2.4. Receptor-interacting protein 140

The receptor interacting protein 140 (RIP140) was originally identified and characterized as a coactivator that interacts with ER and enhances ER-mediated transcriptional activation in an estrogen-dependent manner in breast cancer cells (77). However, the function of RIP140 in NR-dependent regulation of gene expression is far more complex since several subsequent studies have demonstrated that RIP140 binds to and inhibits the transcriptional function of several NRs including TRs, PPARs, RARs, RXRs, GR and AR in the presence of the receptor

cognate ligand/agonist (79-81,144,145). The structure of RIP140 (Figure 5) consists of 10 leucine-rich motifs (nine LXXLL motifs and a tenth LYYML motif) that allow its interaction with the LBD of NRs (78,102,146) and four autonomous repression domains (RD1-4) that are implicated in recruitment of histone- or DNA-modifying enzymes capable of repressing transcription (147).

RIP140 binds to nuclear receptors in a ligand-dependent manner by a mechanism similar to that of the p160/SRCs. The LXXLL motifs of RIP140 dock into a cleft formed by the AF-2 of NRs upon hormonal ligand binding (78,102). Individual LXXLL motifs of RIP140 allow the coregulator to exhibit selective binding to NRs (146,148,149). Particularly, the coregulator LXXLL-less motif 10 interacts preferentially with retinoid receptors (146). Moreover, RIP140 NR boxes also exhibit differential bindings to NRs as either constitutive or ligand-induced binding (150).

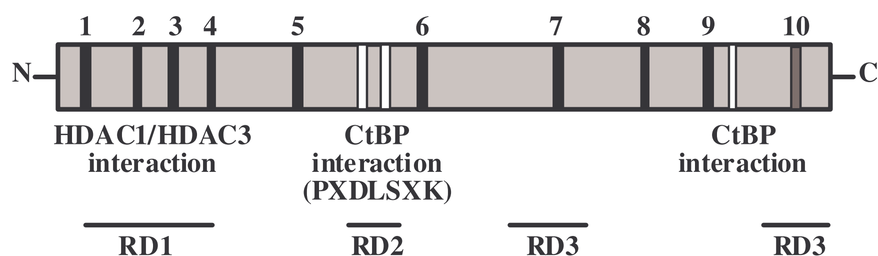

Figure 5. Schematic illustration of RIP140 with its functional domains (edited from (151)). RIP140 is composed of 1158 amino acids, contains nine LXXLL motifs (1-9, black bars) and one LXXLL-less motif (10, the dark grey bar). The coregulator also possesses four repression domains (RD1-4). The regions responsible for binding to selected proteins (histone deacetylase HDAC; C-terminal binding protein CtBP) are also indicated. The white bars represent the interaction sequence/motif for the CtBP.

Evidence has indicated that RIP140 represses transcriptional activity of NRs by competing with coactivator proteins for binding to the receptor AF-2 domain. RIP140 antagonized SRC-1-mediated coactivation of NRs (81). The repressive effect of RIP140 on GR transactivity was partially overcome by an increased expression of TIF2 (144). In addition, Chen and coworkers (152) demonstrated that RIP140 strongly competed with pCAF for ligand-dependent interaction with RAR/RXR both *in vitro* and *in vivo*. Furthermore, studies employing ChiP assays demonstrated a rapid recruitment of RIP140 followed by a delayed recruitment of pCAF to RAR- and TR-target promoters in cells after treatment with retinoic acid (153).

RIP140 also acts as a negative regulator of NR transcriptional function through recruiting chromatin-deacetylating HDACs. The coregulator RD1 has been reported to bind to HDAC 1 and 3 of class I (154,155) and class II HDAC5 (156), while RD2 binds to the C-terminal binding protein (CtBP) that then recruits HDAC enzymes (157,158). Disruption of interaction with CtBP has been shown to result in loss of transcription repression by RIP140 (157). In addition, it has been observed that the ability of RIP140 to inhibit the transcriptional activity of retinoic acid receptors in transfected cells is reversed by treatment with trichostatin A (TSA), a HDAC inhibitor (154). Moreover, the presence of RIP140 has been demonstrated to be required for retinoid acid-induced recruitment of HDACs to ligand-bound RAR/RXR and associated with a decrease in histone acetylation on the promoter of RAR as well as TR target genes (150,155).

RIP140 is detected in most tissues albeit localized in specific cell types where its expression is controlled hormonally or developmentally (159-161). High expression levels are found in the gonads (160) and metabolic tissues including adipose tissue, liver and muscle, where the highest level of RIP140 mRNA is found in WAT, followed by skeletal muscle, BAT and liver (161). During the follicle maturation process, RIP140 expresses increasingly from no detectable expression in the granulosa cells of the primary follicles to high expression in the outer mural cells of the pre-ovulatory follicles (160). The expression level of RIP140 is also significantly increased during the process of adipocyte differentiation (161,162). Hormones that regulate RIP140 expression include estrogen (163), retinoid acid (164), progestin (165), and vitamin D (166).

Consistent with its expression profile, studies with RIP140 ablation in mice or RIP140 depletion by siRNA in cell models have revealed important roles of the coregulator in female reproduction and metabolic regulation (reviewed in (151,167)). Briefly, female mice devoid of RIP140 exhibit complete infertility due to failure of ovulation (159). RIP140-null mice have lower body weight in both males and females as a result of reduced fat accumulation in WAT, resistance to diet-induced obesity and hepatic steatosis and increased insulin sensitivity upon challenging with high fat diet (161,168). Analyses of gene expression in adipocytes from RIP140-null mice have shown that RIP140 suppresses expression of several genes encoding key proteins involved in energy metabolism in adipocytes (169), as well as in catabolic pathways for carbohydrates and fatty acids such as tricarboxylic acid cycle, glycolysis, fatty acid oxidation, oxidative phosphorylation and mitochondrial biogenesis (168).

3. Regulation of nuclear receptor coregulators

NRs and coregulators are subjected to posttranslational modifications (phosphorylation, acetylation, methylation, sumoylation and ubiquitination) or degradation processes which modulate their transcriptional function (reviewed in (64,170-174)). Posttranslational modifications may regulate the coregulator enzymatic activity, its interaction with NRs and/or other transcription factors, its protein stability and subcellular localization (Figure 6). Degradation processes modulate coregulator availability for the interaction with NRs. Regulation of coregulators is implicated as a mechanism for controlling NR-dependent gene transcription in response to cellular signaling.

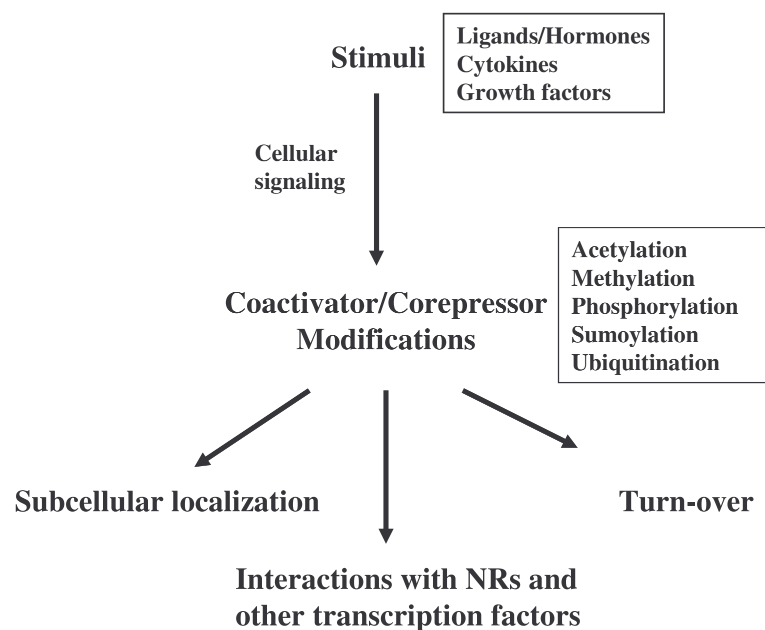

Figure 6. Posttranslational modifications of coactivators and corepressors. Coactivators and corepressors can be postranslationally modified by acetylation, methylation, phosphorylation, sumoylation and ubiquitination in response to extracellular signals. Posttranslational modifications regulate the subcellular localization of coregulators, their interactions with NRs and other transcription factors, and their turn-over via degradation.

3.1. Intracellular signaling pathways

Eukaryotic cells possess many protein kinases that mediate the cellular responses to extracellular stimuli such as hormones. Among them are the cAMP-dependent protein kinase/Protein kinase A (PKA) (175) and the members of the mitogen-activated protein kinase (MAPK) family such as the extracellular signal-regulated kinases (ERKs) and the p38 MAPKs (reviewed in (176)). PKA mediates cellular responses to an elevated intracellular level of cAMP (see section 3.2 for more details). The kinases of the MAPK family are activated by several stimuli including growth factors and cytokines (176). PKA as well as

MAPKs have been implicated in the regulation of NR transcriptional activity mainly by phosphorylation of the receptors (reviewed in (14,170,171)).

3.2. The cAMP/PKA signaling pathway

The cAMP/PKA signaling pathway is activated by a number of hormones such as the pituitary glycoprotein hormones (ACTH, FSH, LH, and TSH (thyroid stimulating hormone)) (177,178), glucagon and adrenaline. The hormones bind to ligand-specific G protein-coupled receptors (GPCRs) located at the plasma membrane and activate adenylyl cyclase (AC) which catalyses the conversion of ATP to the second messenger cAMP. As a result, the intracellular level of cAMP is increased, followed by activation of several downstream kinase signaling cascades including the PKA pathway (Figure 7). For details about the other main targets of cAMP, it is referred to reviews (39,179,180)). The intracellular level of cAMP is controlled by phosphodiesterases which convert cAMP to 5'-AMP.

PKA is the classical effector of cAMP. In an inactive state, the PKA holoenzyme is composed of two regulatory (R) and two catalytic (C) subunits, and is localized in the cytoplasm. There are different isoforms of PKA which are determined by the composition of their regulatory (RIα, RIβ, RIIα, RIIβ) and catalytic (Cα, Cβ, Cγ) subunit isoforms. PKA is activated upon binding to cAMP at its R subunits, which in turn leads to dissociation and release of the C from the R subunits. In a released and free form, the catalytic subunits phosphorylate target proteins and are able to translocate into the nucleus through passive diffusion (Figure 7).

PKA mediates cAMP responses to gene transcription via phosphorylation and activation of the cAMP-response element binding protein (CREB) (181). While the mechanisms for PKA-induced regulation of gene transcription by NRs are still to be elucidated, it has been well established that CREB is the main key mediator of PKA signaling in transcriptional regulation. CREB is phosphorylated by PKA at serine residue Ser-133, which in turn induces transcriptional activation of numerous functional genes important for various cellular processes (182).

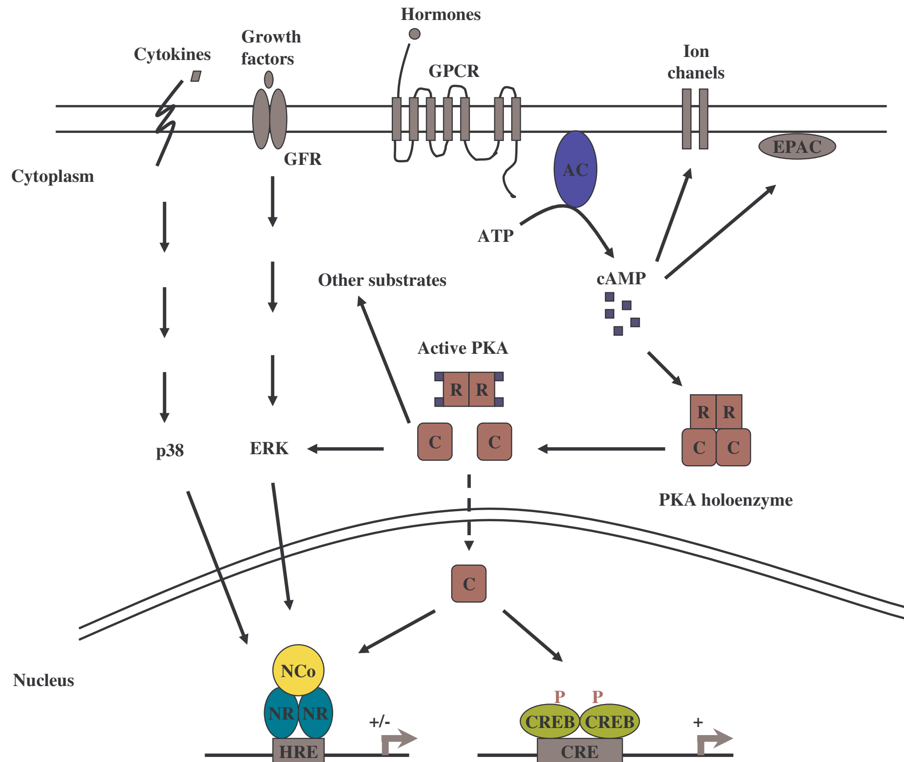

Figure 7. Activation of the cAMP signaling pathway. Hormone binding to the G-protein coupled receptor (GPCR) activates the adenylyl cyclase (AC) which catalyses the conversion of ATP to cAMP. Elevation of cAMP activates PKA and can also modulate other signaling components such as the exchange protein directly activated by cAMP (EPAC) and ion channels. Kinase-active catalytic subunit (C) diffuses into the nucleus, phosphorylates CREB and may modulate NRs and NR coregulators (NCo). Simplified signaling cascades induced by growth factors and cytokines via MAPKs (p38 and ERK) which may be modulated by PKA and are implicated in regulation of NRs and coregulators are also included. (151,176,179,183,184).

3.3. Regulation by protein phosphorylation

Phosphorylation has been observed on coactivator proteins, especially the p160/SRCs, in responses to growth factors, steroid hormones, cytokines, and activation of different protein kinase pathways (reviewed in (185)). SRC-1 possesses at least seven phosphorylation sites (Ser 372, 395, 517, 569, 1033, 1179 and 1185) with consensus sequences for the serine threonine-proline directed family of protein kinases as well as ERKs of the MAPK family (186). SRC-1 can be phosphorylated *in vitro* by ERK-2 (186) and elevation of intracellular cAMP level in cells has been shown to induce phosphorylation of SRC-1 at two mitogen-activated protein kinase (MAPK) sites, threonine 1179 and serine 1185 (187). GRIP1/SRC-2 is phosphorylated at a specific site Ser-736 *in vitro* by ERK and *in vivo* by p38 of the MAPK family in response to EGF (188,189). *In vitro* phosphorylation of GRIP1 by c-Jun N-terminal kinase 1 through sites other than Ser-736 and Ser-554 has also been observed (188).

Similarly, studies have identified AIB-1/SRC-3 as an *in vivo* phosphoprotein with six phosphorylation sites (Thr-24, Ser-505, 543, 857, 860 and 867) (190,191). Several protein kinases including MAPK (190,191), PKA (191), the receptor tyrosine kinase HER2 (human epidermal growth factor receptor 2) (192,193), as well as inhibitor kappa B kinase (IKK) of the cytokine TNF-α (the tumor necrosis factor α (TNF-α) signaling pathway (143,191) have been demonstrated to phosphorylate SRC-3. Phosphorylation induced on other NR coactivator proteins including CBP/p300 by the mitogen-activated/extracellular response kinase kinase 1 (MEKK1) and AMP-kinase (194,195), and PGC-α by p38 kinase (196) have also been observed.

In many cases, phosphorylation of coactivators have been shown to induce an enhancement in gene transcription mediated by NRs (143,186-190,194,196). For example, phosphorylation of SRC-1 increases its ability to coactivate progesterone- or cAMP-dependent transcriptional activation of PR-target genes (187), and interleukine-6-dependent transactivity of AR (197). Mutation of the Ser-736 phosphorylation site of GRIP1 to Ala was observed to decrease EGF-induced GRIP1 coactivation function in PR- and ER-mediated gene transcription (188,189), while mutational disruption of SRC-3 phoshorylation was demonstrated to reduce SRC-3 activity on ER, AR as well as NF-kappa B (191). Conversely, phosphorylation of coactivators can negatively regulate their function, as evidenced by the observation demonstrating that phosphorylation of p300 on Ser-89 by the protein kinase Cδ reduces its coactivity as well as its HAT activity (198). Thus, phosphorylation of coactivators seems to play an important role in mediating cellular signaling to regulation of transcriptional responses of NR-target genes. In addition, it has been proposed that posttranslational modifications provide a molecular determinant for the ability of p160/SRCs to distinguish among different NRs and other transcription factors to allow specific responses to distinct upstream signaling pathways (185).

Although the actual underlying mechanism for regulation of NR-mediated gene transcription by coactivator phosphorylation remains to be elucidated, certain evidence has indicated that physical or functional interactions of coactivators with NRs and other transcription factors are affected by phosphorylation events. Mutational disruption of selected MAPK-phosphorylated sites (Ser 1179 and 1185) of SRC-1 leads to a reduction in its interaction with p300/CBP-associating factor (p/CAF) and a loss of its functional association with CBP (187). MAPK activation increases interaction of AIB-1/SRC-3 with p300 and associated histone acetyltransferase activity (190). Importantly, phosphorylation of SRC-3 selectively affects its ability to interact with steroid receptors (ER, AR) and CBP, but not

CARM1 (191). AMP kinase-induced phosphorylation of p300 dramatically reduces its interaction both *in vitro* and *in vivo* with a range of NRs including PPARγ, TR, RAR and RXR (195). Coactivator phosphorylation may also influence its subcellular localization, as supported by the observation that TNFα-induced phosphorylation of SRC-3 correlates with an increased nuclear import of SRC-1 in cells (143). This is confirmed by another observation where treatment of serum-starved cells with growth factors or phorbol esters, which can phosphorylate p/CIP (SRC-3) (190,192,193), promotes p/CIP translocation into the nucleus (199). Likewise, cytokine or lipopolysaccharide-induced phosphorylation of PGC-1α by p38 kinase leads to an increase in stability and activity of the coactivator (196). Taken together, it seems that phosphorylation of NR coactivators regulates various aspects of their function, including their protein-protein interactions, stability and subcellular localization.

Corepressor proteins have also been observed as targets for phosphorylation elicited by MAPK-mediated signaling pathways (200-202). A proteomic analysis of RIP140 has identified 11 phosphorylation sites of which 5 sites are located in the N-terminal repression domain RD1 (202). Further characterization of the RD1 phosphorylation, which is induced by MAPK, and mutational analyses of the phosphorylation sites in this domain have indicated that phosphorylation enhances RIP140 repressor function through increased recruitment of HDACs (202,203). Another study has reported that phosphorylation of RIP140 regulates its interaction with the 14--3-3 protein, which is able to export the coregulator out of the nucleus and alter its intranuclear localization, resulting in an enhancement of GR-induced transactivation (204). The corepressor SMRT is found as phosphoprotein *in vivo*, and may be phosphorylated by several protein kinases including the MEK1, MAPK and MEKK1 kinases, resulting in its dissociation from the NRs, relocation from nucleus to the cytoplasm, and a reduction of the corepressor function (200,201). Similarly, phosphatidylinositol-3-OH kinase/Akt1 kinase-dependent phosphorylation of N-CoR complex results in a temporally correlated redistribution of N-CoR to the cytoplasm (205). The nuclear to cytoplasm shift of RIP140 or SMRT and N-CoR mediated by phosphorylation may contribute to positive regulation of NR-mediated gene expression by reducing the level of corepressor proteins in their functional compartment. Interestingly, it has been shown that N-CoR is non-responsive to MEKK1-induced phosphorylation and all of the subsequent forms of regulation detected on SMRT, but it responses similarly as SMRT to other downstream signal transduction pathways mediating the effects of EGF/cytokines (201). This suggests that NR coregulator proteins may be differentially selected for regulation by particular signaling pathways.

3.4. The ubiquitin proteasome-mediated degradation pathway

The ubiquitin-proteasome proteolytic pathway represents a major cellular process for selective and specific degradation of proteins in eukaryotic cells. The process involves two discrete and successive steps: i) covalent attachment/modification of the substrate with multiple ubiquitin molecules and ii) degradation of the modified protein by the 26S proteasome complex. The biochemical steps and the components involved in these processes have been reviewed in detail elsewhere (206). Following is a brief description of the pathway.

3.4.1. The ubiquitination system

Ubiquitin (Ub) is a highly evolutionarily conserved polypeptide of 76 amino acids. Conjugation of Ub molecules to target proteins/substrates (S), known as ubiquitination, is a multi-step process which involves three different types of enzymes (Figure 8). Ubiquitin is activated in an ATP-dependent manner by the ubiquitin-activating enzyme E1, to generate a high-energy thiol ester intermediate E1-S-Ub. The activated ubiquitin moiety is then transferred to one of the several ubiquitin carrier/conjugating enzymes E2 (UBCs) forming another high-energy thiol ester intermediate E2-S-Ub. The E2 enzyme then transfers the ubiquitin to a lysine residue in the target substrate/protein which is specifically bound to a member of the third enzyme E3 family named ubiquitin-ligase. The ubiquitin transfer from E2 to the substrate occurs either directly of indirectly depending on the type of the substrate-bound E3 enzyme (206). A polyubiquitin chain is synthesized and elongated by successive attachments of activated ubiquitin molecules to an internal Lys residue of the previously substrate-conjugated ubiquitin moiety. This process seems to be promoted by an ubiquitin chain elongation factor called E4 (207). The polyubiquitin chain-conjugated substrate is recognised by the downstream 26S proteasome complex and targeted for degradation (Figure 8). *In vitro* evidence has suggested that targeted substrates need to be conjugated with polyubiquitin chains of at least 4 ubiquitin moieties in length to be recognised and degraded by the 26S proteasome (208).

More E2 than E1 and even more E3 enzymes have been identified in eukaryotes, as so a single E1 enzyme activates ubiquitin for an array of E2 ubiquitin conjugating enzymes, and each E2/UBC is able to catalyse ubiquitin transfer reaction to substrates for a number of E3 enzymes. Eleven ubiquitin carrier/conjugating enzymes in yeast (Ubc1-8, 10, 11, 13) (206) and over 40 different potential UBCs in the human genome (designated as UbcH followed by variable numbers) (209) have been identified. Although there are several hundreds of heterogeneous E3s (209), many of them can still be classified into two major groups named

the HECT (Homologous to the E6-associated protein/E6-AP C Terminus) domain- and the RING (Really Interesting New Gene) finger-containing E3s. E2/UBC enzymes transfer the activated ubiquitin indirectly to the substrate of the HECT domain E3 by first attaching the ubiquitin to the E3 which subsequently transfers the ubiquitin to the substrate. In contrast, the RING finger E3 ligases catalyse a direct transfer of the activated Ub moiety from the E2-S-Ub intermediate to the E3 ligase-bound substrate. In addition, the identification of E4 enzyme has defined a novel protein family, designated as U box, which is a derivative of the RING finger E3 family (reviewed in (206)).

3.4.2. The 26S proteasome system and protein degradation

The 26S proteasome system is a multisubunit protein complex which is highly conserved among all eukaryotes. The complex is composed of a 20S catalytic core particle sandwiched between two 19S regulatory particles (Figure 8). The 19S regulatory particle contains at least 18 different subunits and possesses ubiquitin hydrolysis activity. This particle serves multiple roles in regulation of the proteosomal activity including substrate selection, substrate preparation for degradation, translocation of substrate into the 20S core, and dictation of the end products after digestion of the substrate protein. In mammalian cells, another regulatory complex named 11S (or REG) regulator or PA28 which binds to the ends of the proteasome core 20S and activate its peptidase function in protein antigen presentation has been identified (210). The 20S has a barrel-shaped structure which is made up of four rings (two α and two β rings) of seven subunits each. While the α-rings are non-catalytic and facing outside, the two inner β-rings have proteolytic active sites with different specificities facing inward into a sequestered proteolytic chamber. With this structure, the 20S proteasome core is able to block random degradation and provide a high level of substrate specificity (206).

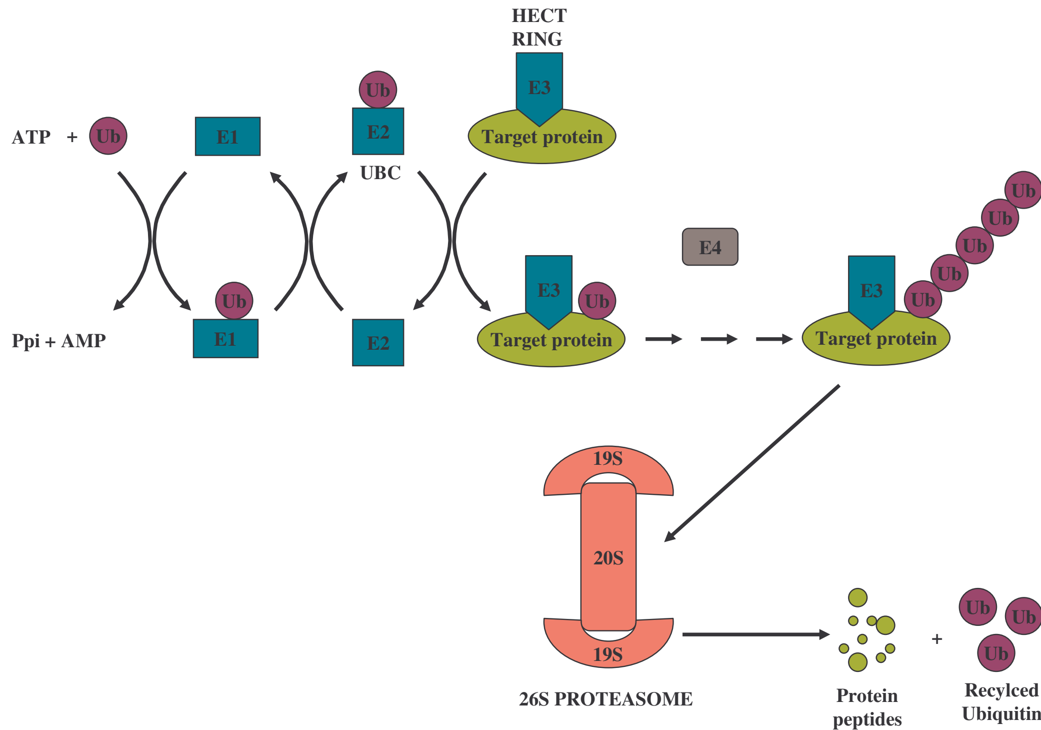

Figure 8. The ubiquitin proteasome-mediated degradation pathway. Ubiquitin (Ub) is activated in an ATP-dependent manner by an ubiquitin activating enzyme E1. The activated Ub moiety is then transferred to one of several ubiquitin- carriers or conjugating enzymes (UBC or E2). A specific ubiquitin ligase E3 (HECT or RING) which is bound to the target protein catalyzes direct or indirect transfer of the activated ubiquitin molecule to the target protein. Polyubiquitination is achieved through successive additions of activated ubiquitins to the previously attached ubiquitin moiety. Polyubiquitinated proteins are recognized and degraded by the 26S proteasome complex. The attached Ub moieties are released and recycled (206).

3.4.3. Signals and modes of substrate recognition

The specificity for substrate recognition and degradation by the 26S proteasome is determined by the E3 ubiquitin ligase alone or in combination with the E2 conjugating enzyme (206). Based on accumulated data, several modes of specific substrate recognition by E3 ligases have been reported (reviewed in (206). Some ubiquitin ligases recognise and bind to their protein substrates simply via the substrate NH_2-terminal residue which is, in this case, referred to as the “destabilizing residue”. Likewise, a number of protein substrates of the ubiquitin-proteasome proteolytic pathway are subjected to phosphorylation before their ubiquitination. This modification in certain cases is necessary for direct recognition of the modified protein by an appropriate E3 ligase. Particularly, several target proteins are recognised indirectly by their specific E3 ligases via ancillary proteins which bind to target proteins. For example, GR is degraded after complex formation with the carboxyl terminus of Hsc70-interacting protein (CHIP) and the chaperon proteins Hsp70 and Hsp90 (211). It has also been noted that many

rapidly degraded proteins contain short hydrophilic stretches of at least 12 amino acids enriched in proline, glutamic acid, serine, and threonine (known as PEST motifs), which in several cases seem to contain phosphorylation sites required for degradation (212). In addition, the destruction box represented by the consensus sequence **R**-A/T-A-**L-**G-X-I/V-G/T-N (absolutely conserved residues are marked in bold) is an important degradation signal for certain proteins especially cell-cycle regulators. Thus, degradation signals are inherent in the primary protein structure of the substrate as specific sequences or motifs which can be modified and/or recognised directly or indirectly by the E3 ligase.

3.5. Regulation by ubiquitination and degradation

The role of the ubiquitin-proteasome pathway as an integral component in NR-mediated gene transcription has been discussed since several proteins/enzymes associated with the ubiquitin-proteasome degradation pathway were identified as coactivators (such as UbcH5 and 7, E6-AP and RPF1/NEDD4 of E3 enzyme group, and subunits of the 26S proteasome complex) or corepressor (*e.g.* the ubiquitin ligase BRCA1) of NRs (reviewed in (213,214). Several reports have demonstrated that liganded-NRs (ER, GR, PR, TRs, PPARγ, RARs and RXRs) and unliganded-NRs (AR, VDR and PPARα) are ubiquitinated and degraded by the ubiquitin-26S proteasome pathway (reviewed in (213,214)). In certain cases, treatment with proteasome inhibitors has been observed to lead to an increase in the receptor level, but inhibition of the receptor transcriptional activity (215-217). This signifies that receptor turnover is required for its efficient transcription. Furthermore, enzymes of the ubiquitin-proteasome pathway are recruited to the promoters of NR-target genes (120,218,219). This strengthens the importance of the ubiquitin-proteasome pathway in transcriptional regulation by NRs.

Regulation of NR coactivators and corepressors through proteasomal degradation has been documented. GRIP1 was first found to colocalize with components of the 26S proteasome including the 20S proteasome core, PA28α and ubiquitin in discrete nuclear foci (220). Later, the coactivator together with the other two members of the p160 coactivator family, SRC-1 and RAC3/SRC-3, as well as CBP have been demonstrated to be targets for degradation via the ubiquitin-proteasome pathway (215,220,221). However, degradation of individual p160/SRCs is stimulated by different UBC enzymes *in vitro* (221). SRC-1 is degraded by UBC2. Likewise, TIF2/GRIP1 degradation requires UBC5, 7 and 8, and degradation of RAC3/SRC-3 is dependent on UBC2, 3, 4 and 5. Interestingly, a recent study has reported that the steady-state level or turn-over of SRC-3 is also regulated by the REGγ-proteasome (20S) machinery in an ubiquitin- and ATP-independent manner (222). REGγ

which is a proteasome activator of the 11S family known to stimulate the trypsin-like activity of the 20S proteasome interacts with and enhances degradation of SRC-3, leading to modulation of the ER-dependent transcription activity. Another example of regulation of coactivators by the ubiquitin proteasome-mediated degradation pathway is the observation that p/CAF stability is decreased by the E3 ubiquitin ligase MDM2 of the RING domain family through ubiquitination and protein degradation (223). Similar effects of the proteasomal pathway on NR corepressor proteins including N-CoR have also been reported (224).

Ubiquitin proteasome-mediated degradation has been proposed as a mechanism for selectively removing corepressors and/or coactivators after they have fulfilled their roles, clearing the way for subsequent recruitment of other coregulators to the promoter-bound NR, and allowing transcription reinitiation and attenuation (65). In line with this, the transducin β-like 1(TBL1) and its homologous protein TBLR1, which are components of the E3 ubiquitin ligase complexes, have been shown to essentially participate in gene transcription by liganded NRs by serving as specific adapters for the recruitment of the proteasome components, mediating the dissociation of the N-CoR corepressor complex and its subsequent degradation (225). This process is believed to mediate a required exchange of corepressors for coactivators upon ligand binding. Moreover, proteasomal degradation of NRs and their coregulators could induce dissociation of the initiation complex and facilitate the assembly of a productive elongation complex (226,227).

3.6. Regulation by other posttranslational modifications

Coactivators and corepressor are also subjected to regulation through other forms of posttranslational modifications. Protein acetylation by transcriptional coactivators possessing acetyltransferase activity including the p160/SRCs and CBP/p300, as well as protein methylation by methyltranferases CARM1 and PRMT1 in NR-mediated transcription was originally directed solely toward histones. However, an increasing number of reports have demonstrated that coactivators are able to posttranslationally cross-modify one another resulting in changes in coregulator association with NRs. Particularly, p300/CBP has been reported to acetylase ACTR/SRC-3 on lysine residues adjacent to an NR box, leading to disruption of the association between this p160 coactivator and the promoter-bound ER and subsequent attenuation of gene transcription (228). Acetylation of RIP140 by p300/CBP which reduces RIP140 interaction with CtBP has also been observed (157). By this observation, the authors have proposed that acetylation-mediated disruption of corepressor

interaction may serve as a mode of transcriptional activation. A recent proteomic analysis has identified eight acetylated lysines in RIP140 that might be important for the coregulator repressive function and subcellular localization (203). While CBP is able to induce acetylation on several coregulator partners, its own coactivator function has been found to be regulated through methylation by CARM1 (229,230). In one case, CARM1 has been shown to methylate an arginine (Arg) residue within the KIX domain of CBP, leading to inhibition of its interaction with the transcription factor CREB (cAMP-response element binding protein) (229). This is thought to increase the CBP pool available for interaction with NRs and therefore could promote CBP coactivation of NR activity (64). In another case, CARM-1-induced methylation of CBP at arginine residues Arg-714, 742 and 768 in another conserved region has been reported to be essential for the cooperative coactivator function of CBP with GRIP1 on NRs as well as for steroid hormone-induced gene activation (230). Interestingly, a recent study by O'Malley group (231) has demonstrated that the p160 member SRC-3 is also methylated by CARM1 at an arginine located in its CARM1 binding region, and this methylation is associated with inhibition of ER-dependent transcription via promoting dissociation between SRC-3 and CARM1.

Coregulators are also regulated by sumoylation which does not promote coregulator protein degradation, but seems to modulate coregulator protein-protein interaction and localization. The NR-interaction domain of both GRIP1 and SRC-1 can be modified by sumoylation (232,233). GRIP1 sumoylation sites have been shown to be essential for its colocalization with AR in the nucleus after testosterone treatment and for its coactivator function on AR-dependent gene transcription (232). Likewise, sumyolation of SRC-1 has been demonstrated to enhance PR-SRC-1 interaction and prolong the retention of SRC-1 in the nucleus (233).

AIMS OF THE PRESENT STUDY

The purpose of this work was to characterize the role of selected nuclear receptor coregulator proteins with a special focus on protein-protein interactions and cAMP-dependent protein kinase signaling pathways. The main objectives of the present thesis were:

1. To characterize the roles of the receptor-interacting protein (RIP) 140 in regulation of Steroidogenic factor 1 (SF-1)-mediated transcriptional activation

2. To identify interactions between SF-1 and nuclear receptor coactivators, and to examine the role of cAMP-dependent protein kinase in regulation of the Steroid receptor coactivators TIF2/GRIP1 and p/CIP

3. To characterize the roles of cAMP and cAMP-element binding protein (CREB) in the regulation of TIF2/GRIP1 coactivator function.

GENERAL DISCUSSION

1. Regulation of SF-1-dependent gene transcription

Since its isolation in the early 1990s by two independent laboratories (10-13), the orphan nuclear receptor SF-1 has emerged more and more evident as an essential regulator of endocrine development and function at multiple levels of the hypothalamic-pituitary-steroidogenic axis. Particularly, SF-1 regulates the transcriptional expression of several pivotal enzymes involved in the steroid hormone biosynthesis (14). The number of genes potentially regulated by SF-1 is rapidly growing. However, knowledge of the molecular mechanism underlying the regulation of gene expression by SF-1 is still limited. The basic molecular mechanism for activation and regulation of steroid hormone receptors or many of the non-steroidal NRs such as TR, RXR and RAR, has been relatively well characterized (6). Ligand-binding is essential for initial activation of these NRs, and the magnitude of their transcriptional activities relies on their virtual interaction with coactivator or corepressor complexes (62,63,234). Ligand-binding leads to changes in NR conformation which allows the NR to recruit coactivator complexes or exchange corepressors with coactivators necessary for transactivation of target genes (62,64). Less has been known about the association between orphan NRs, particularly SF-1, and coregulator proteins.

1.1. Regulation of SF-1 by RIP140

RIP140 has been reported to interact with SF-1 as well as DAX-1 and represses both the basal and cAMP-induced transactivity of SF-1 from the promoter gene encoding human Steroidogenic acute regulatory protein (StAR) (235). Our findings (Paper I) have demonstrated that RIP140 also interacts with SF-1 in an AF-2 dependent manner. The interaction involves multiple LXXLL motifs of RIP140, of which the region containing four C-terminal LXXLL motifs exhibits the strongest interaction. A similar case has also been observed with PPARγ whose interaction with RIP140 is mediated predominantly by multiple LXXLL motifs in the C-terminal 728 amino acids of RIP140 (81). However, both the amino-terminal and carboxyl-terminal fragments of RIP140 interact equally well to GR (204). Preferential use of LXXLL motifs by RIP140 for the interaction with other NRs (RAR, RXR and TR) has also been reported (146,149,150). Together, these studies support the fact that the number and the sequences including the flanking sequences of LXXLL core motifs contribute to the affinity and selectivity of RIP140 for different NRs (148).

RIP140 appears to function as a repressor of SF-1 ((235), Paper I) through different mechanisms. RIP140 represses SF-1 transactivity by antagonizing the stimulatory functions of TIF2 and p/CIP, as well as by association with histone deacetylases (HDACs) as evidenced by the partial inhibition of RIP140 repressive function upon treatment with the HDAC inhibitor TSA (Paper I). The fact that RIP140 acts as a negative coregulator of certain NRs such as PPARγ and GR through competition with the p160/SRCs for receptor binding has previously been reported by others (81,144). Recently, RIP140 has also been reported to compete with p/CAF for binding to RAR (152,153). In addition, RIP140 interacts directly with several HDAC enzymes of both class I and II (154-156), that are able to deacetylate chromatin, leading to transcription repression. While the HDAC activity seems to be necessary for RIP140-mediated repression of RARs and TRs (150,155), HDAC inhibition by TSA has no effects on the active repression of ERα by RIP140 (156). Moreover, RIP140 has been reported to interact with CtBP and disruption of this interaction leads to loss in RIP140- mediated repression of transcription (157). However, whether CtBP is involved in RIP140-mediated repression of SF-1 remains to be investigated.

Our study suggests that the ability of RIP140 to repress SF-1 depends on several factors including promoter contexts, cell types and the expression levels of other coregulator proteins. Different TR-target genes have been demonstrated to recruit different p160/SRCs, each with different temporal recruitment patterns (125). It is likely that SF-1 also requires different sets of coactivators and corepressors to regulate its different target genes. Still, verification of this theory, which may contribute to the understanding of RIP140 function in regulation of different SF-1 target genes in different cells, is needed. We have observed that both RIP140 and TIF2 are expressed at a higher level in Y1 cells than in COS-1 cells. The expression levels of coregulator proteins in different cells may influence the repressive function of overexpressed RIP140. Furthermore, since posttranslational modifications such as phosphorylation and acetylation have been reported to modulate RIP140 activity (157,202-204), it would be of interest to examine the posttranslational status of RIP140 in different cell types subjected to stimulation of different signaling pathways.

1.2. Regulation of SF-1 through interactions with p/CIP and TIF2

SRC-1 has been reported to interact with and potentiate the transcriptional activity of SF-1 through the proximal activation domain (pAF) and AF-2 domain (15). It has also been shown that TIF2/GRIP1 interacts with SF-1 and enhances the transcriptional activation of SF-1 target genes ((51), Paper II). We have demonstrated that p/CIP interacts with and acts as coactivator

for SF-1, and that the nuclear receptor interacting domain of p/CIP with its three LXXLL motifs is required for the SF-1 interaction. This finding has added the nuclear receptor SF-1 as a new member to the list of p/CIP-coactivated NRs that includes ER, GR, PR, TR, RXR, and RAR (103,104,106,236,237). Our results also confirm that all three members of the p160/SRC family stimulate SF-1 activity. A functional redundancy has been observed for other NRs and is believed to act as a safety mechanism in regulation of NR-mediated actions in case one or two of the p160/SRC members are disrupted (107). However, the biological significance of the individual p160/SRCs for SF-1 function remains to be investigated.

The activation function 2 (AF-2) of several NRs is required for their ligand-dependent activation and is the determinant for their interaction with different coregulators (3). Ligand binding leads to changes in the conformation of NRs that form an optimal AF-2 binding surface for coactivators. The AF-2 of SF-1 is required for its interaction with SRC-1 and TIF2 ((15,51), Paper II), as well with p/CIP (Paper II). p/CIP and TIF2 require different amino acids in the AF-2 domain for transcriptional coactivation of SF-1, reflecting different mechanisms of SF-1 binding by these coactivators (Paper II). While the Glutamic acid 454 (Glu454), which is a highly conserved amino acid in the AF-2 domain of NRs (238), is important for p/CIP to stimulate SF-1 transactivity, this residue is dispensable for TIF2-mediated coactivation of SF-1. Different requirements for the conserved Glu residue by other NRs in interaction with coregulators have also been reported (239-241). TR mutated in the conserved Glu shows normal binding affinity for its ligand and normal interaction with the corepressor N-CoR, but is not able to bind to SRC-1 (240). In addition, TIF2 is able to rescue the low ligand-dependent transactivity of a Glu-mutant of AR (241) and the loss of ligand-dependent transcriptional activation by a similar TR Glu-mutant (239). It seems that receptor interactions with different coregulators, particularly the p160/SRCs, are differentially affected by mutation of the conserved Glu residue and the subsequent conformational changes of the receptor. This may represent a mechanism by which NRs interact preferentially and differentially with p160/SRCs (121-123,125), and supports the fact that preferential and differential interactions rely on the LBD structure and the coactivator NR boxes (91,92,121,125-132).

2. Regulation of GRIP1 by the cAMP/PKA signaling pathway

Activation of the cAMP/PKA pathway results in downregulation of GRIP1 through ubiquitination and proteasmomal degradation (Paper III). It has been reported that elevation of cAMP leads to phosphorylation and stimulation of SRC-1 (187). In addition, PKA induce

phosphorylation of SRC-3 and enhances the recruitment of CBP (191). Although our preliminary data do not indicate that GRIP1/TIF2 is phosphorylated directly by PKA (data not shown), a more detailed analysis of GRIP1/TIF2 phosphorylation sites should be performed after stimulation of the PKA pathway. Phosphorylation site prediction of the GRIP1 protein sequence reveals a number of potential phosphorylation sites for PKA, which scatter from GRIP1 N- to the C-terminal regions. Another possibility is that PKA acts through another signaling mechanism such as the MAPK pathway that phosphorylates GRIP1 at Ser-736 (188,189). It should also be noted that GRIP1 is regulated by other posttranslational modifications such as sumoylation (232). Interestingly, acetylation (228) and methylation of SRC-3 (231) have been reported to modulate the SRC-3 coactivator function. Thus, the function of GRIP1 and the effects by PKA may also be related to other modes of modification.

Coactivators of the p160/SRC family are targets for ubiquitination and proteasomal degradation induced by different ubiquitin conjugating enzymes (220,221). Since coactivators have been shown to associate and disassociate on promoters of NR-target genes in a dynamic, orderly and cyclic manner during transcriptional activation by NRs (118,119,173), it is conceivable that they are subjected to degradation. This would allow coactivators to exchange on the promoter and facilitate the transcription process. In certain cellular contexts, selective regulation of specific coactivators by degradation via the ubiquitin-proteasome pathway such as observed for GRIP1 in our report (Paper III) may modulate the relative intracellular levels of coactivators, and therefore may also mediate the remodeling of coactivator networks associated with the NRs.

The functional effects of PKA-mediated degradation of GRIP1 on specific NRs remain to be investigated. As several hormones activate PKA (177,178), we have proposed that PKA-mediated degradation of GRIP1 may represent a mechanism by which PKA-activating hormones can regulate the transcriptional activity of specific NRs, such as GR which has been shown to interact preferentially with GRIP1 (128). The transcriptional activity of GR has been reported to be regulated by cross-talk between glucocorticoids and the cAMP signaling in different extents depending on the promoter context (242-244). In one case, activation of the cAMP signaling by 8-Br-cAMP was shown to antagonize both the hormone-induced and basal transcription of GR target genes through inhibiting the interaction of general transcription factors with the promoter (244). Whether activation of cAMP modulates recruitment of GRIP1 to GR target gene promoters is presently not known. Furthermore, since coactivators of the p160/SRC family seem to functionally compensate for each other

(108,136), selective degradation of GRIP1 by activated PKA could lead to competition between different NRs for interaction with the limited pool of SRC-1 or SRC-3. This may result in global changes of NR-induced gene transcription in the cell, allowing a complicated coordinated cellular response to specific cAMP/PKA signaling.

Modulation of the subcellular localization of coactivators may affect their availability for interaction with NRs. GRIP1 has been found to be localized in the nuclear compartment in a heterogeneous distribution pattern from uniform in nucleoplasm to highly concentrated in subnuclear foci ((101,220,245,246) and Paper III). We have demonstrated that activation of PKA leads to changes in the intranuclear distribution of GRIP1 and to the formation of subnuclear foci that are associated with components of the 20S proteasome and the promyelocytic leukemia (PML) protein. PML is known to be distributed in speckles in the nucleus and is associated with proteasomal degradation of ubiquitinated proteins (220,247,248). Studies have demonstrated that GRIP1 colocalizes with several NRs in subnuclear foci (245,246,249). Interestingly, Black and colleagues (250) have observed that ligand-induced activation of AR alters GRIP1 distribution from subnuclear foci to a more uniform nucleoplasmic pattern and they have proposed that subnuclear foci represent recruitment sites where AR interacts with its coactivators including GRIP1 and CBP prior to transcription. After this engagement at the foci, the coactivator-AR complex then relocates to the promoters and enhances transcriptional activation of target genes (250). Colocalization of GRIP1 with other transcriptional coactivators including CBP and pCAF in subnuclear foci has also been observed (220,250,251). However, it remains to be shown whether PKA activation modulates colocalization of GRIP1 with these coactivators as well as with NRs.

Protein modification with ubiquitin molecules for targeting to proteasomal degradation is highly selective and specific. The selectivity and specificity rely on recognition signals which can be inherent and/or modified from the target protein and is determined by E3 ubiquitin ligases alone or in combination with E2 ubiquitin conjugating enzymes (206). Protein phosphorylation, binding to another protein (ancillary protein) as well as specific sequence motifs inherent in the protein primary structure such as destruction boxes or PEST sequences are among the ubiquitination and degradation recognition signals which have been observed primarily on transcription factors involved in inflammation and immune responses (T cell receptors, nuclear factor kappa-B) and proteins of cell cycle regulation (cyclins) (206,212). Still, little is known about the molecular mechanisms by which NRs, coactivators and corepressors are targeted to ubiquitination and proteasomal degradation, although an increasing number of these proteins have been demonstrated to be targets for the degradation

pathway. Phosphorylation of AR by the phosphatidylinositol 3-kinase (PI3K)-Akt pathway has been reported to promote ubiquitination and degradation of the receptor through a proteasome-dependent pathway (252). Likewise, binding of the carboxyl terminus of Hsp70-interacting protein (CHIP) to GR has been shown to induce its ubiquitination and proteasomal degradation (211). Analysis of mouse GRIP1 and human TIF2 protein sequences by others (220,221) and by us (Paper III) has revealed three potential PEST motifs which could be involved in PKA-mediated targeting of GRIP1 to ubiquitination and degradation. However, our results do not indicate that these motifs are required for PKA-induced degradation of GRIP1 (Paper III). This suggests that other inherent sequence motifs of GRIP1 may act as degradation signals. It is possible that ubiquitination and degradation is signaled upon a complex coordination of several events including phosphorylation and/or other posttranslational modifications as well as interaction with other proteins. A recent report has demonstrated that during ligand-dependent activation of RARα, SRC-3 is phosphorylated by MAPK that leads to an initial potentiation of RARα-dependent transcription, but subsequently to inhibition of the transcription by promoting SRC-3 degradation (237). In addition, the mechanism of target recognition by E2 and E3 enzymes appears to be relatively distinct between different target proteins. While ubiquitination and degradation of SRC-2/TIF2 are induced by UBC5, 7 and 8, SRC-3 and SRC-1 are ubiquitinated and degraded by UBC2, 3, 4 and 5, and UBC2 respectively (221). It would therefore be interesting to investigate whether UBC5, 7 and 8 are involved in the PKA-mediated ubiquitination of GRIP1, at least whether activation of the cAMP/PKA modulates the activity of these enzymes. The E3 ubiquitin ligases MDM2 of the RING-finger protein family are required for phosphorylation-induced ubiquitination and degradation of AR by Akt kinase (252,253). However, which E3 ligase that is specific and/or required for the PKA-dependent ubiquitination and proteasomal degradation of GRIP1 is still unknown.

3. Regulation of GRIP1 by CREB

In Paper IV, we have demonstrated that CREB mediates degradation of GRIP1, and that CREB is required for the PKA-activated degradation of GRIP1. The mechanism by which CREB leads to degradation of GRIP1 and the exact relation to the cAMP/PKA signaling pathway is unclear. We have observed that CREB which is mutated at the PKA phosphorylation site (CREBS133A), as well as a CREB deletion mutant lacking the KID domain, also induce GRIP1 degradation. The results suggest that CREB may regulate GRIP1 through other mechanisms than phosphorylation at Ser-133. It has been reported that the

cAMP/PKA signaling cross-talks with a variety of other signaling pathways including the MAPK pathway (183,254). Furthermore, it has been shown that GRIP1 is regulated by the ERK and p38 of the MAPK signaling pathway (188,189). One possibility is that PKA regulates GRIP1 by interacting with another signaling pathway to stimulate phosphorylation of CREB at other sites than Ser-133. As all the identified putative phosphorylation sites for protein kinases including ERK and p38 MAPK, protein kinase C and B, and the Ca^{2+}-calmodulin dependent kinase are located in the KID domain of CREB (182), our results do not to support this hypothesis. CREB is subjected to several other posttranslational modifications such as acetylation, ubiquitination, sumoylation and glycosylation that regulate its transcriptional activity (255-257). Whether any of these modifications of CREB is induced upon PKA activation and is implicated in the PKA-mediated downregulation of GRIP1 remains unknown.

Protein-protein interaction may induce proteasomal degradation (211). Interaction networks associated with CREB and GRIP1 are obviously complicated and regulated depending on the intracellular environment and exposure to extracellular signals. It is well established that CREB recruits and interacts with CBP/p300 upon activation of PKA (258). The interaction with CBP may serve as an important bridge for the interaction between CREB and NRs, as observed in the case of AR whose transactivity is stimulated after PKA activation (259). Furthermore, it has been reported that CREB physically interacts with CARM1 in a cAMP-dependent manner in hepatocytes (260). Since GRIP1 can bind to multiple NRs and recruit both CBP/p300 and CARM1 to interaction (107), it is conceivable that CREB associates with GRIP1 under certain circumstances. In this study we have demonstrated that CREB interacts directly with GRIP1. Thus we propose that CREB is involved in the PKA-stimulated proteasomal degradation of GRIP1 protein via its direct interaction. This may represent a novel mechanism by which CREB and the cAMP/PKA signaling pathway act to downregulate GRIP1.

CONCLUSIONS

Nuclear receptor coregulators are proteins that play central roles in promoting gene expression by nuclear receptors. This thesis adds important new knowledge to the coregulators that regulate the nuclear receptor Steroidogenic factor 1 as well as a number of other nuclear receptors. Based on the results presented in this thesis the following conclusions were drawn.

1. The nuclear receptor coregulator RIP140 interacts with SF-1 in an AF-2 dependent manner, and several domains of RIP140 are involved in the protein-protein interaction. The carboxyl-terminal region that contains 4 of its 9 LXXLL motifs shows the strongest interaction with SF-1. RIP140 acts as a potent corepressor of SF-1, and inhibits a number of SF-1 responsive promoter genes. Histone deacetylase activity may partially explain the RIP140-mediated repression of SF-1 and competition by coactivators (p160/SRCs) counteracts the RIP140-mediated inhibition of SF-1 dependent transcription.
2. The coactivators p/CIP and TIF2/GRIP1 interact directly with SF-1 through its AF-2 domain. Both coactivators stimulate SF-1 dependent transcription. While activation of the cAMP/PKA signaling pathway potentiated p/CIP-mediated coactivation of SF-1, cAMP/PKA impaired the ability of TIF2/GRIP1 to stimulate SF-1 mediated transactivation of different reporter genes in COS-1 and adrenocortical Y1 cells. Activation of cAMP/PKA also inhibits the TIF2/GRIP1 coactivation of a number of other nuclear receptors. Our results demonstrate that extracellular signals that activate the cAMP/PKA pathway differentially regulate coactivators of the p160/SRC family.
3. Activation of cAMP/PKA signaling pathway leads to ubiquitination and proteasomal degradation of GRIP1. cAMP/PKA also regulates the intracellular distribution of green fluorescent protein-GRIP1, and stimulates the recruitment of GRIP1 to subnuclear foci that are colocalized with proteasome components.
4. Degradation of GRIP1 induced by PKA activation depends on the transcription factor CREB, but phosphorylation of CREB at Ser-133 by PKA is not required for the CREB-mediated degradation of GRIP1. A novel protein-protein interaction between GRIP1 and CREB is identified, which may represent an important part of the mechanisms underlying GRIP1 degradation.

FUTURE PERSPECTIVES

Our results suggest that RIP140 negatively regulates SF-1 transactivity probably through competition with the p160/SRCs for SF-1 binding and through recruitment of HDACs. This can be subjected to further studies on the recruitment of HDACs or other corepressors such as CtBP during repression of specific SF-1-target genes. Furthermore, the fact that RIP140 coregulator function varies depending on the promoter contexts and cell type raise an interesting question of whether RIP140 is differently regulated in these different cellular contexts. Of note, the RIP140 expression levels were shown to be different in COS-1 versus Y1 cells. A study of posttranslational modifications of RIP140 in different cell types and/or under different cellular signaling would be relevant to answer the question.

TIF2 and p/CIP coactivator functions are differentially regulated upon activation of PKA signaling. To understand the molecular mechanism underlying this differential regulation, characterization of posttranslational modifications of TIF2 and p/CIP upon activation of PKA should be performed. Moreover, a recent ChIP-based investigation has showed slightly different patterns of TIF2 and p/CIP recruitment to SF-1 target gene upon ACTH activation (57). A similar ChIP-based study which includes both basal and PKA-activated condition would be of significance since this could verify *in vivo* the important roles of p160/SRC coactivators for NR basal transactivity via interaction and may also reveal how different TIF2 and p/CIP are recruited to NRs when the cAMP/PKA signaling is activated.

One of the main focuses of this study is regulation of coregulator proteins by signals acting through the cAMP/PKA signaling pathway. To elucidate the molecular mechanism underlying this regulation, several questions should be answered, such as which signals the cAMP/PKA induces to target GRIP1/TIF2 to ubiquitination and proteasomal degradation. Mapping of GRIP1 domains that are required for its PKA-mediated downregulation may reveal an inherent sequence motif that acts as recognition signal and site for ubiquitination. Furthermore, it would be interesting to perform proteomic characterization of possible posttranslational modifications of GRIP1 induced upon activation of PKA, and to identify possible components of the ubiquitin-proteasome degradation pathway that is associated with GRIP1. The identification of CREB as a phosphorylation-independent negative regulator of GRIP1, a required mediator of PKA-induced degradation of GRIP1, and an interaction partner of GRIP1 has shed light on how PKA might regulate GRIP1. Mapping of CREB domains that are required for the interaction between GRIP1 and CREB should be characterised.

Adipogenesis and hepatic gluconeogenesis are cellular processes that are regulated by cAMP signaling. Altered regulation of adipogenesis is associated with obesity whereas hepatic gluconeogenesis is related to increased fasting plasma glucose and type 2 diabetes (261,262). CREB has been shown to modulate expression and transcriptional activity of the key regulators of these processes, including the nuclear receptor PPARγ and its coactivator PGC-1α (263,264). In addition, it has been demonstrated that transgenic mice deficient in CREB exhibit fasting hyperglycemia and have a fatty liver phenotype (264). Interestingly, ablation of SRC-2/GRIP1 also leads to significant downregulation of hepatic genes involved in energy metabolism (141). In other words, reduction in SRC-2/GRIP1 is associated with decreased energy storage and increased energy utilization. One might speculate whether GRIP1 and its regulation by the cAMP/PKA pathway and CREB could play roles in adipogenesis and hepatic gluconeogenesis. Characterisation of the mechanisms underlying CREB-mediated regulation of GRIP1 could provide important insights in the understanding of biological functions of GRIP1 in energy metabolisms and obesity.

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