Role of the 26S proteasome and Posttranslational Modifications in Regulating the Expression of Retinoic Acid-Responsive Genes

By

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CONTRIBUTIONS OF CO-AUTHORS

The manuscript which comprises appendix C was written by Dr. Qiao Li. All the research described in this thesis is my own work except for the following:

1) Luciferase assay and quantitative real-time RT-PCR in figure 4.1 A & B were performed by Mahmoud Abed.

2) The colocalization immunofluorescent images in figure 4.10 A were picked up from Dr. Jihong Chen experiments.

ABSTRACT

Retinoic acid (RA) has been recognized as a chemotherapeutic agent for various malignances such as lung, skin as well as cervical cancers. It binds to retinoid receptors heterodimers and consequently activates several RA-responsive genes which are involved in many biological processes including vertebrate development, bone growth, vision, haematopoiesis, cell growth, differentiation and apoptosis. These genes are under the control of numerous regulators to ensure their timely ordered activities. Among these regulators, we focused here on the 26S proteasome and ubiquitination.

It has been reported that the activity of the ubiquitin/proteasome system (UPS) plays a fundamental role in retinoic acid receptor (RAR)-regulated transactivation. The mechanisms underlying this role, however, remain to be established. Chromatin immunoprecipitation (ChIP) assays in our study demonstrated that the 26S proteasome activity is important for preserving the occupancy of a TATA box-containing RA-responsive promoters by liganded retinoid receptors and thus by their coactivators. Additionally, by using coimmunoprecipitation assays and by measuring the half-life of retinoid receptors, we found that the non-proteolytic function of the proteasome is required for ligand-dependent association between DNA-free RAR-α and both DNA-free RXR-α and coactivators. Moreover, using immunofluorescent staining and *in vivo* ubiquitination assays, a proteasome inhibition-dependent cytoplasmic localization of RAR-α as well as ligand-enhanced ubiquitination and stabilization of RAR-α were shown.

Our findings therefore, define novel mechanisms by which the UPS controls RAR-regulated genes. Furthermore, we shed new light on the regulators of retinoid receptors ubiquitination and subcellular localization.

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AD	Alzheimer's Disease
AF-1 or 2	Activation Functions 1 or 2
ALS	Amyotrophic Lateral Sclerosis
AMP	Adenosine Monophosphate
AP1	Activating Protein 1
APC	Adenomatous Polyposis Coli
APL	Acute Promyelocytic Leukemia
AR(s)	Androgen Receptor(s)
ARG1	Argininosuccinate synthetase 1
AR-JP	Autosomal Recessive-Juvenile form of PD
at-RA	All trans-Retinoic Acid
ATP	Adenosine Triphosphate
AZ	Antizyme
BSA	Bovine Serum Albumin
^{o}C	Degree Centigrade
CBP	CREB Binding Protein
CD9	Cluster of Differentiation 9
CDK(s)	Cyclin-Dependent Kinase(s)
cDNA	coding DNA
ChIP	Chromatin Immunoprecipitation
ChIP-seq	ChIP-sequencing

CHX	Cycloheximide
c-IAP1	cellular-Inhibitor of Apoptosis Protein 1
CKI(s)	Cyclin-dependent Kinase Inhibitor(s)
c-myc	cellular-myelocytomatosis
CO2	Carbon dioxide
CoA	Coenzyme A
CP	Core Particle
CRABP I or II	Cellular Retinoic Acid Binding Protein I or II
CRBP I or II	Cellular Retinol Binding Protein I or II
CRE	Cyclic AMP-Response Element
CREB	CRE-Binding protein
Ct	Cycle threshold
CTL(s)	Cytotoxic T Lymphocyte(s)
Cyp26A1	Cytochrome P450 26A1
DBD(s)	DNA Binding Domain(s)
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DR(s)	Direct Repeat(s)
DTT	Dithiothreitol
E6-AP	E6-associated protein
EC	Embryonal Carcinoma

ECL	Enhanced Chemiluminescence
EDTA	Ethylene Diamine Tetra Acetic
ENaC	Epithelial Na^+ Channel
ER	Endoplasmic Reticulum
ER(s)	Estrogen receptor(s)
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GR(s)	Glucocorticoid Receptor(s)
H	Histone
HAT(s)	Histone acetyltransferase(s)
HCl	Hydrogen Chloride
HDAC(s)	Histone deacetylase(s)
HIF-1α	Hypoxia-inducible factor-1α
His-tagged	Hexa histidine (6xHis)-tagged
Hox	Homeobox
HPV	Human papilloma virus
HRP	Horseradish peroxidase
hrs	hours
HTLV-I	Human T-lymphotropic virus Type I
IE	Immediate early
IgG	Immunoglobulin G
IκB	Inhibitor kappa B

INF-γ	Interferon γ
IP(s)	Immunoprecipitation(s)
kDa	Kilo-Daltons
LBD	Ligand binding domain
LH	Luteinizing hormone
LiCl	Lithium chloride
Luc	Luciferase
LXR(s)	Liver X receptor(s)
M	Molar
Mdm2	Murine double minute 2
µCi	micro curie
µg	microgram
MHC class I	Major histocompatibility complex class I
µl	micro litre
µM	Micro molar
ml	milli litre
mM	milli molar
mRNA	messenger RNA
MSK1	Mitogen- and stress-activated protein kinase
Na^+	Sodium Ion
NaCl	Sodium chloride
NAD(P)	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium Bicarbonate

nM	nano molar
N-CoR	Nuclear receptor corepressor
NFκB	Nuclear Factor Kappa B
NP-40	nonyl phenoxypolyethoxylethanol-40
NQO1	NAD(P)H quinone oxidoreductase 1
NR(s)	Nuclear receptor(s)
ODC	Ornithine decarboxylase
PA28 or 200	Proteasome activator 28 or 200
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-Buffered Saline
PBS-T	PBS-Tween
PCAF	p300/CBP-associated factor
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
p38MAPK	p38 Mitogen-Activated Protein Kinase
PML	Promyelocytic leukemia
PMSF	Phenyl Methyl Sulfonyl Fluoride
PPAR(s)	Peroxisome Proliferator-Activated receptor(s)
PR(s)	Progesterone receptor(s)
PTMs	Posttranslational modifications
p-value	probability-value
PVDF membranes	Polyvinylidene fluoride membranes
PY	Proline-rich (PPxY)

RA	Retinoic Acid
Rad6	Radiation sensitive 6
RALDH	Retinal Dehydrogenase
RAR(s)	Retinoic Acid receptor(s)
RARE(s)	RA response element(s)
RBP	Retinol binding protein
RE(s)	Response Element(s)
REs	Retinyl esters
RNA	Ribonucleic Acid
RNA Pol II	RNA polymerase II
ROH	Retinol
ROHDH	Retinol dehydrogenase
RP	Regulatory particle
rpm	revolutions per minute
Rpn	Regulatory Particle, non- ATPase-like
Rpt	Regulatory Particle, ATPase-like
RSV-β-Gal	Respiratory syncytial virus β-Galactosidase
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR(s)	Retinoid X receptor(s)
S	Sedimentation coefficient
^{35}S	^{35} Sulfer
SAGA	Spt-Ada-Gcn5-acetyltransferase
SCAs	Spino-Cerebellar Ataxias

SCF_{Skp2}	Skp1/Cul1/F-box protein
S.D.	Standard Deviation
SDS	Sodium Dodecyl sulfate
SF-1	Splicing factor-1
SMAD	Sma and Mad related proteins
SMCC	Srb and mediator protein-containing complex
SMRT	Retinoid and thyroid hormone receptors
Sp1/Sp3	Specificity Protein 1/ Specificity Protein 3
SRC-1/-3	Steroid receptor coactivator-1/-3
STRA6	Stimulated RA gene 6
SUMO-2	Small ubiquitin-like modifier-2
SWI/SNF	SWItch/Sucrose Non Fermentable
$T^1/_2$	Half life
TBL1	Transducin β-like protein 1
TBLR1	TBL1-related protein
TBP	TATA box-binding protein
TCR	Transcription-coupled repair
TE	Tris-EDTA
TFIIH	Transcription Factor II H
TR(s)	Thyroid receptor(s)
TTNPB	4-[(E)-2-(5, 6, 7, 8-Tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid
Ub	Ubiquitin

UPS	Ubiquitin/proteasome system
VAD	Vitamin A deficiency
VDR(s)	Vitamin D3 receptor(s)
VHL	Von Hippel-Lindau
XIAP	X-linked Inhibitor of Apoptosis Protein
XPB	Xeroderma Pigmentosum B

DEDICATION

I dedicate this thesis to:

- ***My father and my mother***

  Who were motivating me during my study since I was a kid and who instilled in me the passion for science at a young age. Also, they taught me the value of persistence and hard working.

- ***My husband***

  Who supported, encouraged and helped me a lot during my PhD study.

- ***My kids***

  Who tolerated me and gave me all the love, joy, smiles and happiness.

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I would not be here without choosing me to continue my post graduate studies in Canada from my department and university back home, "Clinical Pathology Department, Faculty of Medicine, El Minya University, Egypt". As well, I would not be able to be here without the financial support of my sponsorship, "the Egyptian Ministry of High

Education, Egyptian government, Egypt" through "the Graduate Scholarship Doctoral Research Award". Therefore, I would like to sincerely acknowledge all of them.

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CHAPTER 1: REVIEW OF LITERATURE

This thesis deals with a broad topic about the regulators of direct retinoic acid target genes specifically, the ubiquitin/proteasome system (UPS). In order to provide the reader with the essential background to understand the experimental data, and their significances, I will review a number of subjects related to retinoids: **1)** retinoids; what they are, their biological functions, metabolism and therapeutic potential, **2)** signal transduction mediated by nuclear receptors, with emphasis on the retinoid nuclear receptors, **3)** mode1 genes used to analyze retinoid signaling involving RAR-β2 and Cyp26A1 and **4)** P19 cells as a model cell line to study the regulators of retinoic acid-inducible genes. Additionally, I will introduce the UPS and various relevant topics: **1)** the 26S proteasome structure, function, related diseases and inhibitors as well as **2)** ubiquitination as one of the posttranslational modifications and its role in gene transcription control. Finally, I will discuss the relevance of UPS to retinoid signaling.

1.1 Retinoids

Retinoids are either natural metabolites or synthetic derivatives of vitamin A [reviewed in (Sebrell and Harris, 1967)]. Vitamin A and its active retinoid derivatives "vitamin A family of molecules" are involved in many physiological processes. These processes include vertebrate development, embryonic patterning, learning, memory, neurogenesis, reproduction, bone growth, vision, haematopoiesis, immunity, cell differentiation, proliferation and apoptosis [reviewed in (Kastner et al., 1995; Mongan and Gudas, 2007; Olson and Mello, 2010; Wolf, 1984)]. Therefore, the vitamin A family

of molecules is critical for life but in adequate doses and at specific time points. This is strongly supported by a number of diseases related to abnormalities in the normal levels of these molecules: either vitamin A deficiency (VAD) or hypervitaminosis A [reviewed in (Hathcock et al., 1990; Underwood and Arthur, 1996)].

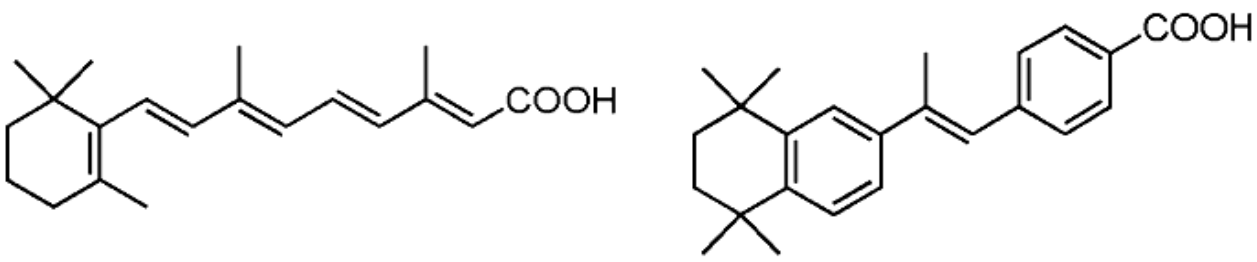
1.1.1 Retinoids metabolism

Vitamin A is a fat soluble vitamin that is present in diet in the form retinyl esters (REs) or provitamin A carotinoids [reviewed in (Goodman, 1984; Ross, 1993)]. For vitamin A to perform its physiological role it must be metabolized into its biologically active metabolites. Thus reviewing the metabolism of vitamin A and retinoids is important.

In the lumen of the gastrointestinal tract, vitamin A in the form of REs and carotinoids is transformed to retinol (ROH) [reviewed in (Goodman, 1984; Ross, 1993)]. ROH is converted back to REs in intestinal cells, packaged into chylomicrons and then released into lymph and blood streams to be stored in the liver as retinyl palmitate [(Fidge et al., 1968; Smith et al., 1973) and reviewed in (Ross, 1993)]. In the blood stream, ROH is associated with retinol binding protein (RBP) [reviewed in (Ross, 1993)]. To achieve the biological functions of retinoids, ROH molecules have to enter its target cells through diffusion or through binding to RBP receptor, STRA6 [(Kawaguchi et al., 2007) and reviewed in (Ross, 1993)]. Inside the cells, cellular retinol binding proteins (CRBP) I and II bind to ROH. Intracellular ROH is converted to retinal and then to all-trans retinoic acid (at-RA) using retinol dehydrogenase (ROHDH) and retinal dehydrogenase (RALDH) respectively. The at-RA must transferred to the nucleus to mediate its effects where it binds to nuclear retinoid receptors [reviewed in (Ross, 1993)].

Finally, at-RA is catabolized via the Cyp26 family of enzymes after performing its physiological roles to control the cellular levels of retinoids and hence prevent their uncontrolled activity (Reijntjes et al., 2007).

In vivo, there are different metabolites of retinoic acid (RA) which are either biologically active or inactive. The active derivatives include at-RA (the most potent derivative of vitamin A) (fig. 1.1) (Dunagin et al., 1964) and 9-cis-RA (Heyman et al., 1992; Levin et al., 1992). These derivatives are responsible for the biological functions of RA. In contrast, the inactive derivatives such as 4-hydroxy RA (Skare et al., 1982) and 4-oxo RA (Eckhoff et al., 1991) are the resultants from at-RA catabolism by Cyp26 enzymes. In this study, we used arotinoid acid, 4-[(E)-2-(5, 6, 7, 8-Tetrahydro-5, 5, 8, 8-tetramethyl-2-naphthalenyl)-1-propenyl] benzoic acid "TTNPB" to induce RA-responsive genes. **TTNPB** is a synthetic derivative of RA which is a highly potent retinoic acid receptor (RAR)-selective ligand (fig. 1.1) (Pignatello et al., 1997).



all-trans Retinoic Acid TTNPB

Figure 1.1 **Chemical structures of at-RA and TTNPB**

Diagramatic illustration of the chemical structures of at-RA and its synthetic derivative TTNPB.

1.1.2 Therapeutic potential of retinoids

Therapeutically, retinoids are well established pharmacological compounds for treating a number of dermatological diseases such as psoriasis and acne [(Orfanos et al., 1997) and reviewed in (Seaton, 2006)]. Moreover, retinoids have shown cancer prophylaxis or chemopreventive potential in clinical trials to prevent the development of second breast cancers, second primary hepatocellular carcinomas and aerodigestive tract tumors [reviewed in (Freemantle et al., 2003; Hong and Sporn, 1997; Nason-Burchenal and Dmitrovsky, 1999; Sun and Lotan, 2002)]. Additionally, retinoids are used to treat preneoplastic lesions such as cervical dysplasia, oral leukoplakia and xeroderma pigmentosum [reviewed in (Hong and Sporn, 1997; Nason-Burchenal and Dmitrovsky, 1999; Sun and Lotan, 2002)]. Retinoids have also been reported to be a promising agent in treating some malignancies alone or in combination with certain chemotherapies such as interferon. These malignancies include mycosis fungoides, lung cancer, Kaposi's sarcoma, high-risk neuroblastoma, advanced renal cancer, skin and cervical squamous cell carcinomas [(Berg et al., 2000; Cheer and Foster, 2000; Lippman et al., 1994; Lippman et al., 1993) and reviewed in (Freemantle et al., 2003; Moore et al., 1994; Reynolds and Lemons, 2001)].

The best example for the anti-carcinogenic potential of retinoids is the dramatic effects of at-RA (tretinoin) in treating acute promyelocytic leukemia (APL). APL is a hematologic malignancy of the white blood cells. The use of RA, in combination with other chemotherapeutics, is already established in treating this form of cancer. The outcome of this drug combination is long term remission in 70% of patients which is a remarkable success [reviewed in (Freemantle et al., 2003; Tallman and Nabhan, 2002)].

1.1.3 Nuclear receptor superfamily

RA exerts its physiological functions through binding to nuclear retinoid receptors which are members of nuclear receptor (NR) superfamily [reviewed in (Leid et al., 1992; Linney, 1992; Mangelsdorf et al., 1995)]. NRs are ligand-dependent transcription factors which up- or down-regulate the expression of a variety of genes [reviewed in (Aranda and Pascual, 2001; Mangelsdorf et al., 1995)]. They are subdivided into two subgroups according to their characters in the resting state i.e. with no ligand-induction [reviewed in (Mangelsdorf et al., 1995; Novac and Heinzel, 2004)]. Also, these receptors can be subdivided into four classes according to their dimerization and their DNA-binding sequences [reviewed in (Mangelsdorf et al., 1995; Stunnenberg, 1993)].

The first class of nuclear receptors is the steroid hormone receptors. In the absence of ligand, steroid hormone receptors are associated with heat shock proteins within cytoplasmic non-functional complexes, so they do not affect basal promoter activity. Moreover, with ligand activation, these receptors bind to inverted DNA repeats as homodimers. This group includes estrogen (ERs), progesterone (PRs), androgen (ARs), glucocorticoid and mineralocorticoid receptors [reviewed in (Mangelsdorf et al., 1995; Tsai and O'Malley, 1994)]. In contrast, the second class of nuclear receptors that consists of retinoic acid receptor (RARs), retinoid X receptor (RXRs), thyroid hormone receptor (TRs), liver X receptor (LXRs), peroxisome proliferator-activated receptor (PPARs) and vitamin D3 receptor (VDRs) binds DNA in the absence of ligands, and has a silencing effect on the basal promoter activity [(Lu et al., 2001) and reviewed in (Mangelsdorf et al., 1995; Tsai and O'Malley, 1994)]. The members of this group of nuclear receptors form heterodimers with RXRs that bind to DNA response elements consisting of direct

repeats. The third class of nuclear receptors includes RXRs homodimers that bind to DNA direct repeats. RXRs homodimers bind DNA in the absence of ligand. Lastly, the members of the fourth class of nuclear receptors which encompasses a number of orphan receptors bind to expanded core half-sites as monomers [reviewed in (Mangelsdorf et al., 1995)].

1.1.4 Nuclear retinoid receptors

Retinoid receptors include RARs and RXRs. Both RARs and RXRs have three different subtypes (α, β, γ) and each one of these subtypes have multiple isoforms. These receptors share a modular domain structure (fig. 1.2) [reviewed in (Chambon, 1996)]. An A/B domain at the N-terminus encloses the intrinsic transcriptional activation function 1 (AF-1) that is ligand-independent activation domain [reviewed in (Warnmark et al., 2003)]. A highly conserved DNA binding (DBD) or C domain that contains two zinc fingers is responsible for receptors/DNA binding and dimerization. The hinge or D domain connects the C domain with the E domain for flexibility [reviewed in (Chambon, 1996)]. The ligand binding (LBD) or E domain is responsible for ligand binding, dimerization as well as coactivator and corepressor binding. Importantly, the E domain encompasses transactivation function 2 (AF2) which is the ligand-dependent activation domain [reviewed in (Warnmark et al., 2003)]. Finally, at the C-terminus there is the F domain that is absent in RXRs, and its function, if any, has not been fully elucidated (fig. 1.2) [reviewed in (Chambon, 1996)].

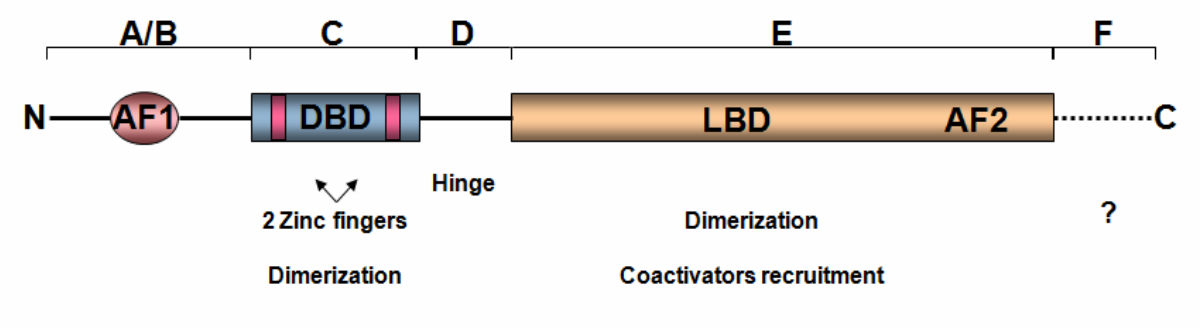


Figure 1.2 **Structural and functional organization of retinoid receptors-specific domains**

The amino-terminal A/B domain contains the AF1, ligand-independent activation domain. The most conserved region is the DNA-binding C domain that is involved in RAR/RXR dimerization. The D domain composes a highly flexible hinge region. The E domain is the ligand binding domain, which encloses the AF2, ligand-dependent activation domain and is responsible for dimerization as well as coregulators binding. Finally, the carboxy-terminal F domain which is not present in RXRs currently has no known function.

The at-RA binds only RARs while 9-cis- retinoic acid binds both RARs and RXRs [(Heyman et al., 1992; Levin et al., 1992) and reviewed in (Allenby et al., 1993)]. The functions of ligand-bound RXRs in the absence of liganded RARs are not well understood. RXR-specific ligand is unable to activate the transcription of RAR/RXR heterodimers-regulated genes. This is called RXR subordination [(Germain et al., 2002; Westin et al., 1998) and reviewed in (Clarke et al., 2004)]. However, several studies demonstrated synergistic effects when liganded RXRs are accompanied with liganded RARs [(Lotan et al., 1995; Schulman et al., 1997) and reviewed in (Clarke et al., 2004)].

RAR/RXR heterodimers bind to specific DNA sequences within the regulatory regions (either promoters or enhancers) of direct RA target genes. These DNA sequences are termed RA response elements (RARE). The classic RARE is composed of two consensus sequences known as direct repeats (DR) of the hexamer motif PuG(G/T)TCA separated by either two or five base pairs (DR2 and DR5) (Leid et al., 1992). The RXR partner of these heterodimers binds the 5' element of RARE (see examples of DR5 RAREs in fig. 1.4) (Kurokawa et al., 1994; Kurokawa et al., 1993; Rastinejad, 2001).

1.1.5 Regulation of RA-responsive gene expression

RA-responsive gene expression is regulated in a ligand-dependent manner (fig. 1.3) [reviewed in (Bastien and Rochette-Egly, 2004; Torchia et al., 1998; Xu et al., 1999a)]. In the absence of ligand, RAR/RXR heterodimers occupy RAREs, and the expression of RA target genes is repressed via recruitment of corepressors such as silencing mediator of retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (N-CoR) (Chen and Evans, 1995; Horlein et al., 1995; Ordentlich et al., 1999). These corepressors recruit other complexes that comprise histone deacetylase (HDAC)

enzymatic activity which maintain chromatin transcriptionally in an inactive state (fig. 1.3 A) [reviewed in (Privalsky, 2004; Urnov et al., 2001)].

Upon ligand induction, RAR/RXR heterodimers that occupy RAREs, undergo conformational changes in their LBD(s) that lead to more association between RAR and RXR, increase in the binding affinity of RAR/RXR heterodimers to RAREs as well as release of corepressors and recruitment of coactivators. There are different transcriptional coactivators with intrinsic histone acetyltransferase (HAT) activity that are involved in the activation of RA target genes including steroid receptor coactivator-1 (SRC-1), CREB (cyclic adenosine monophosphate (AMP)-response element-binding protein) binding protein (CBP), p300, and CBP/p300-associated factor (PCAF) (Näär et al., 2001; Rachez and Freedman, 2001; Rosenfeld and Glass, 2001). These coactivators increase the acetylation of histones within RA target genes core promoters and hence, make this region more accessible for transcription (Bannister and Kouzarides, 1996; Chen et al., 1997; Cowger and Torchia, 2006; Spencer et al., 1997; Yang et al., 1996). Additionally, CBP/p300 connect liganded retinoid receptors/coactivators complexes to RNA polymerase II (RNA Pol II) and to the general transcriptional machinery following their sequential recruitment by Srb and mediator protein-containing complex (SMCC) (Ito and Roeder, 2001). Furthermore, CBP/p300 connect RAR/RXR heterodimers/coactivators complexes that occupy promoter regions to those bound to enhancer regions in a scaffold like function (Chakravarti et al., 1996; Chan and La Thangue, 2001; Xu et al., 1999a). Finally, the SWI/SNF chromatin remodeling complex is recruited to facilitate transcription elongation (fig. 1.3 B) (Flajollet et al., 2007; Kiefer et al., 2004).

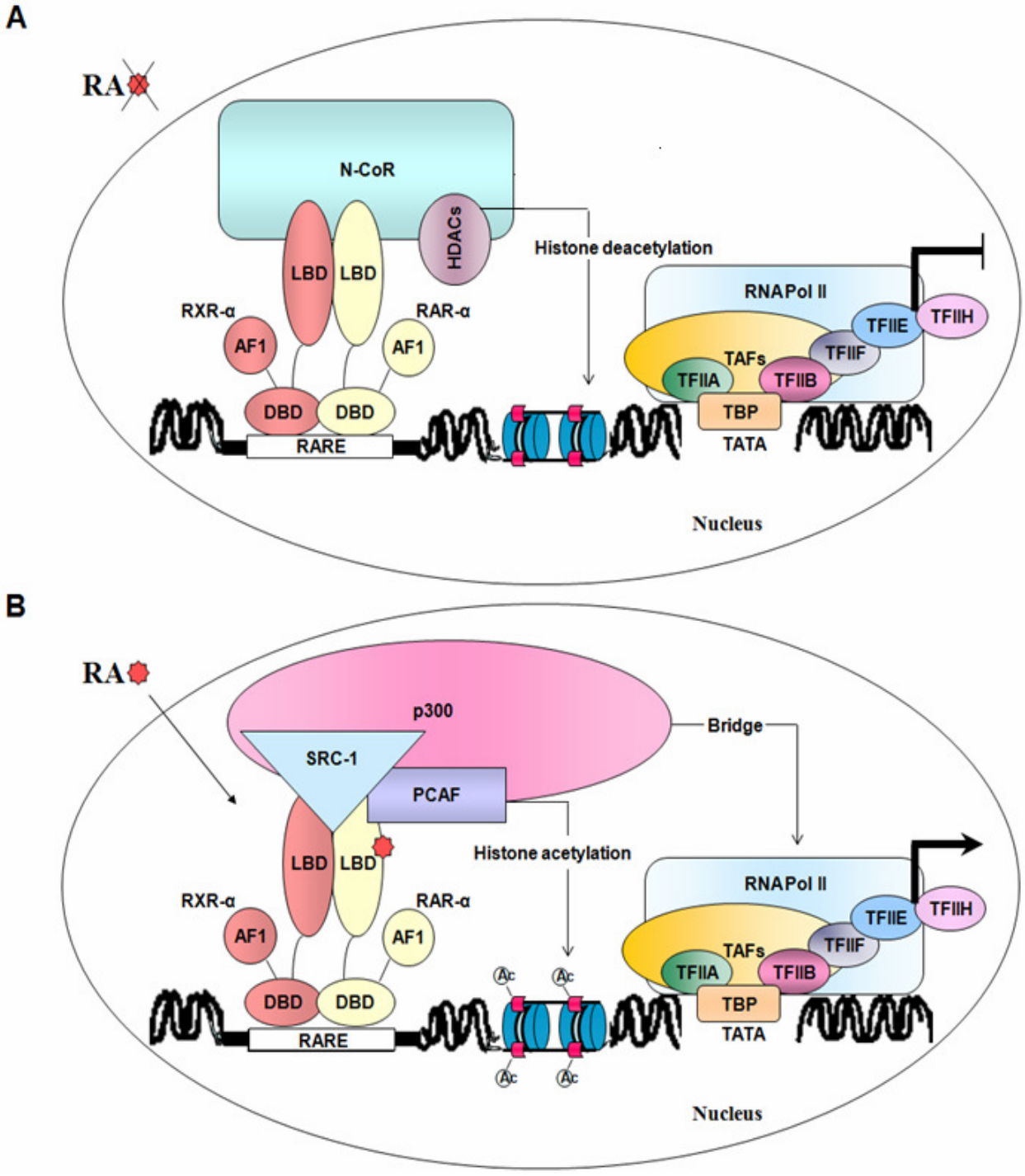

A
RA
N-CoR
HDACs
Histone deacetylation
LBD
LBD
RXR-α
RAR-α
AF1
AF1
DBD
DBD
RARE
RNA Pol II
TFIIE
TFIIH
TAFs
TFIIF
TFIIA
TFIIB
TBP
TATA
Nucleus
B
RA
p300
Bridge
SRC-1
PCAF
Histone acetylation
LBD
LBD
RXR-α
RAR-α
AF1
AF1
DBD
DBD
RARE
Ac
Ac
Ac
Ac
RNA Pol II
TFIIE
TFIIH
TAFs
TFIIF
TFIIA
TFIIB
TBP
TATA
Nucleus

Figure 1.3 **Regulation of direct RA target genes expression**

(A) In the absence of ligand, corepressors such as N-CoR associate with RAR/RXR heterodimer complex that occupies RARE and recruit other corepressors with HDAC enzymatic activities. The histone deacetylase activity helps to maintain the tight association between deacetylated core histones and chromatin which results in repression of gene transcription. (B) Upon ligand binding to RAR/RXR heterodimer complex, conformational changes in the LBDs of these receptors happen, which cause dissociation of corepressors and subsequent recruitment and binding of coactivators such as SRC-1, CBP/p300 and PCAF. These coactivators have HAT activities. The p300 protein functions as a bridge that connect receptors/coactivators complex to RNA Pol II and general transcription factors. Acetylated histones dissociate from the chromatin which in turn facilitates transcription of target genes.

Interestingly, the activity of the 26S proteasome as well as retinoid receptors posttranslational modifications (PTMs) such as ubiquitination, phosphorylation and acetylation had been shown to influence the transcription of RA target genes [(Gaillard et al., 2006; Gianni et al., 2006; Minucci et al., 1997; Perissi et al., 2004) and reviewed in (Bastien and Rochette-Egly, 2004)]. Some of these unconventional coregulators for the expression of these genes will be discussed later in this thesis.

1.1.6 Retinoic acid-responsive genes

RA mediates the expression of a wide variety of genes either directly or indirectly. CRABP I and II, RAR-β, Cyp26 and Homeobox (Hox) family of genes are examples of direct RA-responsive genes as they contain RAREs within their promoters [(de The et al., 1990; Langston and Gudas, 1992; Loudig et al., 2000; Ruberte et al., 1992) and reviewed in (Gudas, 1994)]. For my Ph.D. project, I investigated the regulation of RA-dependent RAR-β2 and Cyp26A1 genes in P19 cells as a model system to better understand direct RA target genes expression.

1.1.6.1 RAR-β2 gene

The RAR-β gene was mapped to chromosome region 3p21–3p24 (Picard et al., 1999; Virmani et al., 2000). Human RAR-β gene has four isoforms, β1, β2, β3 and β4 from which RAR-β2 is the predominant one [reviewed in (Chambon, 1996)]. The transcription of RAR-β2 isoform is modulated by a highly conserved CpG Island-containing P2 promoter as well as alternative splicing (Poulain et al., 2009; Zelent et al., 1991). RAR-β2 gene expression is mediated through RAR/RXR heterodimers that bind to RARE-containing promoters (de The et al., 1990; Dilworth and Chambon, 2001). The

mouse RAR-β2 promoter region is located about 30 base pairs upstream of the transcriptional initiation site and is composed of two conserved DR5 response elements which are categorized according to their proximity to TATA box into proximal (p) RARE and distal (d) RARE (fig. 1.4 A) (de The et al., 1990; Lefebvre et al., 2002b; Sucov et al., 1990; Valcárcel et al., 1994). The DR5 response elements are responsible for a rapid and potent RA-induction of RAR-β2 gene expression [(de The et al., 1990; Hoffmann et al., 1990; Sucov et al., 1990; Vivanco Ruiz et al., 1991) and reviewed in (Mangelsdorf and Evans, 1995; Sanguedolce et al., 1997; Stunnenberg, 1993)]. Additionally, the functional promoter of this gene consists of consensus DNA sequence termed activating protein 1 (AP1) binding site along with cyclic AMP-response element (CRE) which are located at the 5' flanking region of RAREs and TATA box (Kruyt et al., 1992; Lefebvre et al., 2002b; Mitchell and Tjian, 1989; Sanguedolce et al., 1997; Shen et al., 1991; Valcarcel et al., 1997). CRE functions as an enhancer element for the RAR-β2 gene (Wei et al., 2000).

The RAR-β2 gene is a RA-inducible tumor suppressor which is known to be down-regulated or even silenced in many premalignant and malignant cells such as breast, lung, prostate, cervical and ovarian cancers [(Dhillon et al., 2004; Lotan et al., 2000; Widschwendter et al., 1997; Xu et al., 1999b; Xu et al., 1997) and reviewed in (Xu, 2007)]. Furthermore, the resistance of cancer cells to the growth inhibitory effects of retinoids was attributed to the loss of RAR-β2 gene expression (Faria et al., 1999; Gebert et al., 1991; Hoffman et al., 1996; Hu et al., 1991; Xu et al., 1994). Methylation of the RAR-β2 gene promoter and as a result downregulation of RAR-β2 gene expression was detected in hepatocellular carcinoma (Lee et al., 2003; Yang et al., 2003). Moreover, a

hepatitis B virus integration site in hepatocellular carcinomas was demonstrated in the RAR-β2 gene (Benbrook et al., 1988; Dejean et al., 1986).

The RAR-β2 gene plays an important role in regulating a broad array of cellular processes by modulating the expression of different downstream genes either directly or indirectly. These cellular processes include cellular immune response, apoptosis, proliferation, and differentiation (Bain et al., 1994; Roy et al., 1995; Toulouse et al., 2000).

1.1.6.2 Cyp26A1 gene

The Cyp26A1 gene was mapped to human chromosome 10q23-q24 (White et al., 1998). This gene is a member of the cytochrome P450 family of enzymes that is involved in the catabolism of at-RA into 4-hydroxy RA and 4-oxo RA (Abu-Abed et al., 1998; Swindell et al., 1999). A number of studies using animal models or embryonal carcinoma cell lines have categorized Cyp26A1 gene as one of the direct RA target genes. This suggests that retinoid catabolism is regulated by positive feedback signals and that Cyp26A1 gene is fundamentally important for RA homeostasis (Abu-Abed et al., 1998; Iulianella et al., 1999).

The transcription of Cyp26A1 is under the control of two highly conserved DR5 RAREs. The mouse proximal response element (R1) is about 80 base pairs upstream from the transcription initiation site within the promoter region and the mouse distal response element (R2) is about 2000 base pairs upstream from the transcription initiation site (fig. 1.4 B) (Loudig et al., 2000; Loudig et al., 2005). In the presence of low concentrations of RA, RAR/RXR heterodimers that bind the R2 as well as the R1 response regions work synergistically to mediate the induction of Cyp26A1 gene

expression (Loudig et al., 2005). Additionally, the Cyp26A1 proximal promoter contains a G-rich element for Sp1/Sp3 binding (Loudig et al., 2000).

Cyp26A1 null mutations in mouse embryos are embryonically lethal from caudal regression with spina bifida, lumbosacral skeletal muscles malformation, tail truncation and developmental abnormalities of the hindbrain, digestive and urogenital tracts (Abu-Abed et al., 2001; Sakai et al., 2001). Pharmacologically, RA resistance that could be developed in a number of patients with acute promyelocytic leukemia may be attributed to RA induction of Cyp26A1 gene expression (Loudig et al., 2005; Ozpolat et al., 2002).

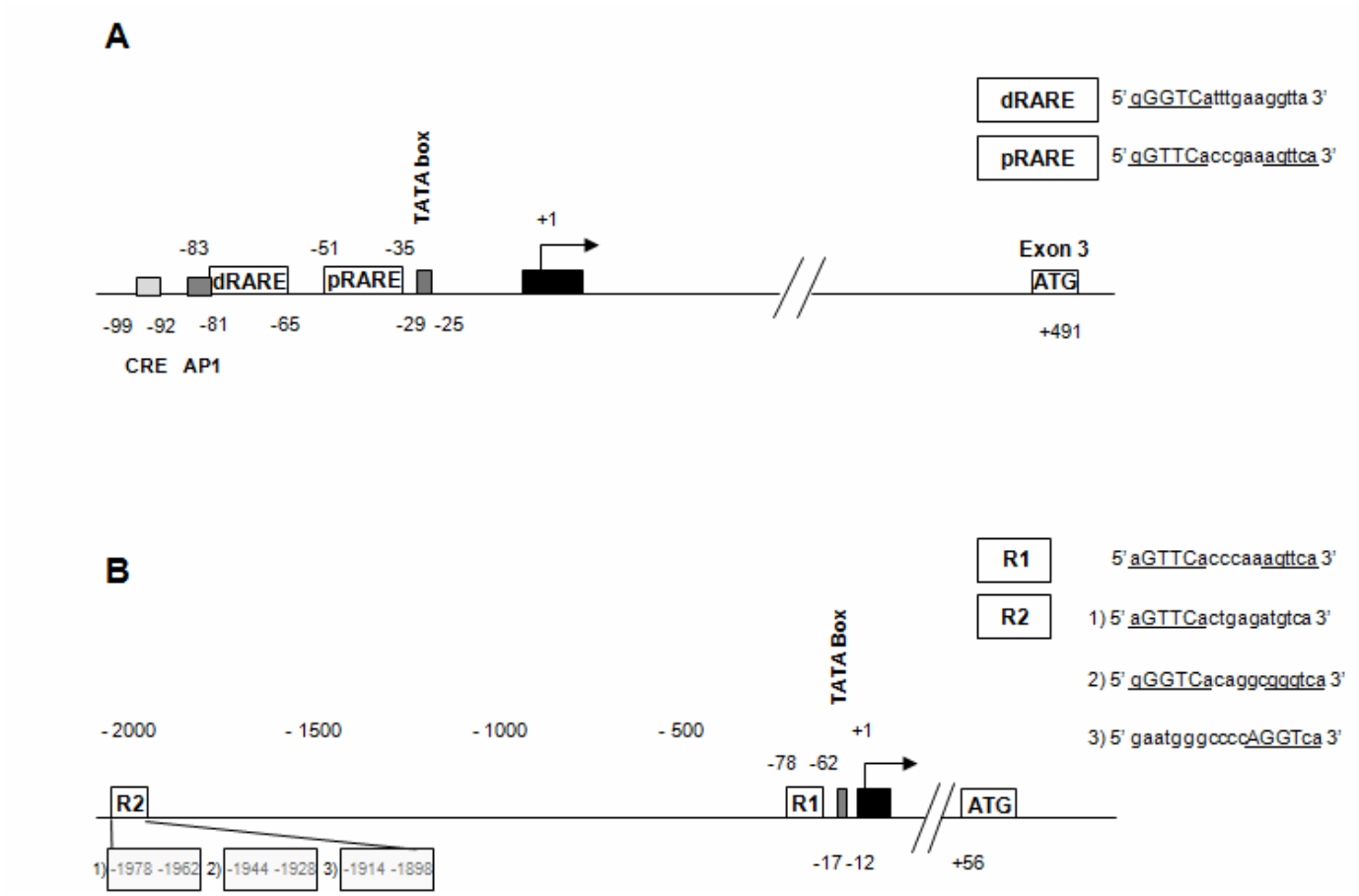

A
dRARE
5' gGGTCatttgaaggtta 3'
pRARE
5' gGTTCaccgaaagttca 3'
TATA box
+1
-83
-51
-35
dRARE
pRARE
Exon 3
ATG
-99 -92 -81 -65 -29 -25
+491
CRE AP1
B
R1
5' aGTTCacccaaagttca 3'
R2
1) 5' aGTTCactgagatgtca 3'
2) 5' gGGTCacaggcgggtca 3'
3) 5' gaatgggcccc AGGTca 3'
TATA Box
- 2000
- 1500
- 1000
- 500
+1
-78 -62
R2
R1
ATG
1) -1978 -1962 2) -1944 -1928 3) -1914 -1898
-17 -12
+56

Figure 1.4 **The DNA regulatory regions of the mouse RAR-β2 and Cyp26A1 genes**

(A) Diagrammatic illustration of the mouse RAR-β2 promoter region. AP1 is activating protein 1 response element and CRE is cAMP response element. The dRARE is in the 5'-flanking region of the pRARE and is separated from it by 13 base pairs. The two response elements are in close proximity to the TATA box. The pRARE is a perfect DR5 sequence however the dRARE is a half site. (B) Diagrammatic illustration of the mouse Cyp26A1 promoter region. The R1 region is close to the TATA box while the R2 region is 2000 base pairs upstream of the TATA box. R1 response element contains one perfect DR5 site but R2 response element includes three DR5 sequences, one of them is perfect and the other two are half sites. ATG is the DNA sequence corresponding to start codons.

1.2 Pl9 cell line

The Pl9 cells are mouse embryonal carcinoma (EC) cells that are originated from mouse teratocarcinomas (McBurney and Rogers, 1982). These cells were created by implanting a 7.5 day female mouse embryo with heterozygous X-linked alleles into an adult mouse testis (McBurney and Rogers, 1982; McBurney and Strutt, 1980; Rossant and McBurney, 1982). Therefore, P19 cells carry an euploid male karyotype (40:XY) (McBurney and Rogers, 1982; Rossant and McBurney, 1982).

P19 cells contain undifferentiated stem cells. Thus, P19 cells are pluripotent cells that can be induced to differentiate into cells of the three germ layers i.e. ectoderm, mesoderm and endoderm (Mummery et al., 1986; Mummery et al., 1985; Mummery et al., 1991; Wang et al., 2006). Moreover, these cells could be maintained in tissue culture dishes as undifferentiated cancer cells [reviewed in (McBurney, 1993)]. Formation of cell aggregates or embryoid bodies is necessary for P19 cells to differentiate. Additionally, the use of RA or dimethyl sulfoxide (DMSO) is also required for the differentiation process to proceed. RA (> 10^{-7} M) potentiates the differentiation of P19 embryoid bodies to neurons, astrocytes and glial cells while RA (< 10^{-7} M) is required for cell aggregates differentiation to mesodermal derivatives. DMSO (0.5-1%) stimulates P19 aggregates differentiation to cardiac and skeletal muscles [(Aizawa et al., 1991; Edwards et al., 1983; Edwards and McBurney, 1983; Jones-Villeneuve et al., 1983; McBurney et al., 1982) and reviewed in (McBurney, 1993)].

Pl9 cells have been used in our study as they represent a useful model for studying the expression of RA-responsive genes. Indeed, these EC cells show a large induction of many RA-responsive genes immediately following RA stimulation. RAR-α and RAR-γ

are constitutively expressed in P19 cells and both of them dimerize with RXRs to mediate RA induction of the RAR-β2 gene via β-RARE but RAR-α is the more predominant retinoid receptors subtype in these cells (Pratt et al., 2000). The RAR-β2 gene was categorized among RA-induced immediate early (IE) genes in P19 cells (de The et al., 1990). Also, Cyp26A1 mRNA levels rapidly increase in P19 cells after RA-induction. Hence, this gene is a RA IE gene similar to RAR-β2 in P19 cells (Loudig et al., 2005; Ray et al., 1997).

1.3 The 26S proteasome

The 26S proteasome is a labile structure that is present in the nucleus and cytoplasm of eukaryotic cells [reviewed in (Wójcik and DeMartino, 2003)]. It is an ATP-dependent protease which is responsible for degrading misfolded, damaged and short-lived regulatory proteins in all eukaryotic cells [reviewed in (Baumeister et al., 1998; Voges et al., 1999)]. The proteasome function is fundamental for growth, development and viability. Different yeast proteasomal mutations are lethal in some instances while others cause global deficiency in proteolysis and defects in stress response. The 26S proteasome has a "dumbbell-shaped" structure with a sedimentation coefficient (S) of 30S and a molecular mass of 2000 kilodaltons (kDa) according to the most precise measurements (fig. 1.5 A) [(Yoshimura et al., 1993) and reviewed in (Coux et al., 1996)]. In the following sections, I will review some topics related to the 26S proteasome.

1.3.1 Structure

The 26S proteasome is a highly conserved multi-subunit structure composed of 20S proteolytic core particle (20S CP) caped from one or two sides by the 19S regulatory

particles (19S RP). The 19S RP is itself subdivided into two subunits, a lid and a base complexes (fig. 1.5 A) (Groll et al., 1997; Lowe et al., 1995).

I- The 20S core particle

The 20S proteasome is a barrel-shaped particle that is responsible for the proteolytic function of the proteasome [reviewed in (Heinemeyer et al., 2004)]. It is composed of four stacked rings which include two outer rings with seven α-subunits in each ring and two central rings that have seven β-subunits per ring (Groll et al., 1997; Lowe et al., 1995). In each β-ring, only three subunits have threonine protease active sites. These active sites include peptidyl-glutamyl peptide-hydrolase (post acidic or caspase-like) (β1), trypsin-like (β2) and chymotrypsin-like (β5) activities [reviewed in (Baumeister et al., 1998; Voges et al., 1999)]. The α-rings are structural in function as they form a gate for the proteolytic core in association with the base of the 19S particle. This gate regulates the access of protein substrates to the interior of the proteolytic cavity and prevents the free or uncontrolled degradation of folded substrates (Kohler et al., 2001; Smith et al., 2007).

II- The 19S regulatory particle

The 19S RP is one of the subunits that associate with the 20S core structure of the proteasome along with the 11S particle, PA28 and PA200 (Ustrell et al., 2002). These subunits are considered activators for the 20S proteasome because they enhance the protease function of the proteasome by regulating the opening of the gate for the 20S CP (Kohler et al., 2001; Smith et al., 2007). The 19S RP is composed of a base and a lid subunits. The lid is formed by eight non ATPase subunits (Rpn-3, -5, -6, -7, -8, -9, -11, -

12) (Sharon et al., 2006). While the function of the lid is crucial for appropriate degradation of polyubiquitinated proteins, the exact role of the lid is not well characterized. This role may be attributed to the presence of receptors in the lid subunit for binding of polyubiquitin chain linked to the target protein substrates that will be subjected to degradation (Sharon et al., 2006). The base subunit has six ATPases (Rpt-1, -2, -3, -4, -5, -6) from the AAA family beside three non ATPase subunits (Rpn-1, -2, -10) [(Zwickl et al., 1999a) and reviewed in (Zwickl et al., 1999b)]. These ATPases interact with the α-rings to form the 20S CP gate and control its opening (Smith et al., 2005). Moreover, the base is responsible for deubiquitinating, unfolding and finally translocating the target protein substrates into the interior of the proteolytic core after opening its gate in the α-rings in an ATP-dependent manner (Lam et al., 2002; Liu et al., 2006; Smith et al., 2005).

1.3.2 The 26S proteasome and protein degradation

The 26S proteasome is the major structure where degradation of eukaryotic intracellular proteins (nuclear, cytoplasmic, endoplasmic reticulum (ER) or mitochondrial) takes place. These proteins include short-lived regulatory enzymes, long-lived (days or weeks) proteins such as actin or myosin as well as proteins with very long half life (months) such as hemoglobin. Damaged, misfolded, aged, unfolded, or oxidized proteins are degraded through the proteasome as well [reviewed in (Coux et al., 1996; Rechsteiner and Hill, 2005; Schwartz and Ciechanover, 2009)]. The protein degradation process by the 26S proteasome is mediated via the ubiquitin (Ub)/proteasome system (UPS) for the majority of cellular proteins in an ATP-dependent manner. However, it has been known that there are some exceptions to this role and proteasomal protein

degradation could happen through ubiquitin-independent pathway both in an ATP-dependent or independent manner [reviewed in (Coux et al., 1996)].

I- Ubiquitin/proteasome system-mediated degradation

The process of eukaryotic intracellular proteins degradation through the UPS has two successive steps: 1) marking target protein substrate by multi-ubiquitination process and 2) proteasomal degradation of the multi-ubiquitinated protein (fig. 1.5 B). Protein ubiquitination involves three enzymatic cascades. Initially, the ubiquitin-activating enzyme E1 activates the C-terminal Glycine of ubiquitin protein in an ATP-dependent reaction. Activated ubiquitin is then transferred to one of many E2 ubiquitin-conjugating enzymes. Finally, E2-ubiquitin delivers ubiquitin to targeted protein substrate in the presence of E3 ubiquitin ligases (Myung et al., 2001). There are hundreds of E3 ligases which are essential for substrate selection (Myung et al., 2001). The steps of protein ubiquitination are repeated for multiple cycles, where at each new cycle a ubiquitin molecule is added to a lysine (K) residue within the ubiquitin molecule from the previous cycle. As a result, a polyubiquitin chain is formed and binds covalently to the target protein (Myung et al., 2001).

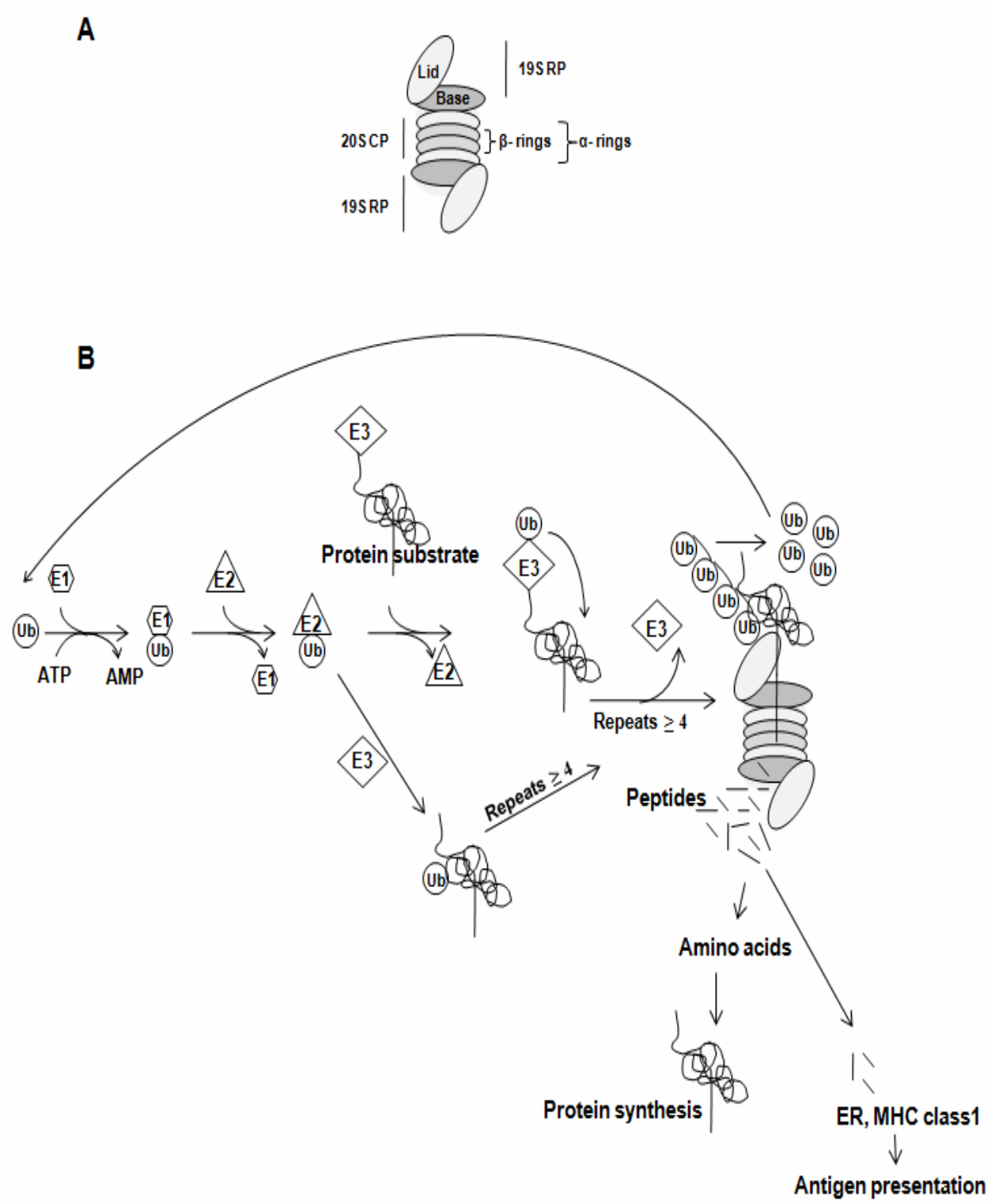

A
Lid
19S RP
Base
20S CP
β- rings
α- rings
19S RP
B
E3
Protein substrate
Ub
E1
E2
ATP
AMP
Repeats ≥ 4
Peptides
Amino acids
Protein synthesis
ER, MHC class1
Antigen presentation

Figure 1.5 **The ubiquitin/proteasome pathway and its role in protein degradation and antigen presentation**

(A) A scheme of the 26S proteasome structure. (B) E1, ubiquitin-activating enzyme activates ubiquitin in an ATP dependent manner. The activated ubiquitin is then transferred to E2, ubiquitin carrier enzyme followed by target protein substrate ubiquitination through E3, ubiquitin ligase. The E3 enzyme may bind to ubiquitin protein then transfer this ubiquitin protein to the protein substrate, or may act as a mediator that is required for direct substrate ubiquitination. Multiple ubiquitination cycles proceed to form a polyubiquitin chain attached to target protein. Polyubiquitin chain binds to the lid of the 19S proteasome then the protein is deubiquitinated and unfolded by the base subunit of the 19S RP before entering the 20S CP to be degraded to small peptides. The resultant ubiquitins are reused again by UPS and the resultant peptides are used either used for protein re-synthesis or for antigen presentation via ER and MHC class I.

II- Ubiquitin-independent/ATP-dependent degradation

Ubiquitin-independent/ATP-dependent degradation refers to degradation of proteins using the whole 26S proteasome structure (ATP-dependent) but without prior ubiquitination. There are many examples that support this type of protein degradation. For example, the enzyme ornithine decarboxylase (ODC) which is a short-lived protein involved in the biosynthesis of polyamine is degraded through the ubiquitin-independent/ATP-dependent pathway after being inactivated by the inhibitory protein, Antizyme (AZ) [(Glass and Gerner, 1987; Rosenberg-Hasson et al., 1989) and reviewed in (Gerner and Meyskens, 2004)]. Moreover, degradation of the tumor suppressor, p53 was thought to be only via the UPS but Asher and colleagues in 2002 reported that this protein may be subjected to ubiquitin-independent/26S proteasomal degradation. This pathway is Mdm2-independent and is stimulated by inhibition of NAD(P)H quinone oxidoreductase 1 (NQO1) (Asher et al., 2001; Asher et al., 2002a; Asher et al., 2002b; Asher et al., 2004; Asher et al., 2003). The cyclin-dependent kinase inhibitor (CKI), $p21^{WAF1/CIP1}$ is subjected to both ubiquitin-dependent and independent proteolysis by the whole 26S proteasome structure. Additionally, its ubiquitin-independent proteolysis can occur through the 20S proteasome only (ATP-independent) (Amador et al., 2007; Chen et al., 2007b; Chen et al., 2004b; Li et al., 2007).

III- Ubiquitin-independent/ATP-independent degradation

The degradation of eukaryotic intracellular protein may also happen through the 20S proteasome in a ubiquitin-independent and ATP-independent manner as there is no 19S RP. The ornithine decarboxylase (ODC) protein was shown to undergo ubiquitin-

independent/ATP-independent degradation. This ODC degradation was mediated after dissociation of ODC-NQO1 complex by NQO1 inhibitors (Asher et al., 2005a). Furthermore, p53 and p73 proteins were shown to undergo Mdm-2- and ubiquitin-independent degradation via the 20S proteasome in a pathway regulated by NQO-1 (Asher et al., 2005b). Also, highly oxidized proteins such as oxidized hemoglobin and insulin-B chain were demonstrated to be subject to ubiquitin-independent/19S RP-independent degradation through the 20S proteasome (Dick et al., 1991; Fagan and Waxman, 1991; Giulivi et al., 1994).

1.3.3 Biological cell functions regulated by the ubiquitin/proteasome system

The process of protein degradation is destructive in nature as indicated by its name but it is critical for cell homeostasis as it maintains the balance between protein synthesis and catabolism. Therefore, the UPS is involved in controlling a number of cellular processes. In the following section, I will review some of these processes and how the UPS participates in their regulation.

1) Cell cycle control

Cell cycle progression is vital for cell proliferation and division (Zavitz and Zipursky, 1997). It is well established that the UPS is involved in cell cycle progression [reviewed in (Barinaga, 1995; King et al., 1996; Murray, 1995)]. The eukaryotic cell cycle is promoted by positive regulators such as cyclins and cyclin-dependent kinases (CDKs) while it is down-regulated by cyclin-dependent kinase inhibitors (CKIs). The progression through the eukaryotic cell cycle requires fluctuation in the activity of CDKs.

CDKs are activated by forming a complex with cyclins. Therefore, cyclin synthesis is involved in the activation of CDKs and their destruction is required for inactivating CDKs [reviewed in (Spataro et al., 1998)]. Both cyclins and CKIs are degraded through the UPS; thus proteasome function is essential for eukaryotic cell cycle control [reviewed in (Murray, 1995; Tyers and Jorgensen, 2000)].

There are various cyclins which require the proteasomal function to deactivate their dependent CDKs after being dissociated from CDK/cyclin complex. Cyclin B, A, D and E are examples of these cyclins which are required by the cell for entry into mitosis, S-phase progression, entry into S-phase of the next cycle, and transition from G1-phase to S-phase respectively (Amon et al., 1994; Bastians et al., 1999; Diehl et al., 1997; Glotzer et al., 1991; Ohtsubo et al., 1995; Singer et al., 1999; Winston et al., 1999).

Additionally, $p21^{Cip1/WAF1}$, $p27^{Kip1}$ and $p19^{INK4d}$ are members of CKIs family which negatively regulate cell cycle progression. These CKIs are ubiquitinated and targeted for degradation by the 26S proteasome. Destruction of $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ is necessary for the transfer of eukaryotic cell cycle from G1-phase to S-phase [(Sheaff et al., 2000; Sutterluty et al., 1999) and reviewed in (Tyers and Jorgensen, 2000)]. $p19^{INK4d}$ degradation is needed for activation of CDK4 and CDK6 by cyclin D which is important for the cell to enter S-phase (Thullberg et al., 2000).

In conclusion, the 26S proteasome has a role in controlling both negative and positive eukaryotic cell cycle regulators. This role includes both enhancement of DNA replication and cell cycle arrest.

2) Organ development and cell differentiation

Cell cycle withdrawal is a crucial step for organ development and terminal cell differentiation (Zavitz and Zipursky, 1997). It was demonstrated that the UPS participates in the process of withdrawal from the cell cycle and hence plays a fundamental role in cellular differentiation [reviewed in (Naujokat and Hoffmann, 2002)]. This role is a complex one, as both positive and negative correlations between the activity of the UPS and cellular differentiation were observed (Baldassarre et al., 2000; Di Cunto et al., 1998; Negishi et al., 2001; Spinella et al., 1999; Urano et al., 1999).

The negative correlation between the activity of the proteasome and cellular differentiation was shown for RA-induced neuronal differentiation of human embryonal carcinoma cells through proteasome inhibition experiments. Moreover, inhibition of rat osteoblast differentiation by transforming growth factor β1 has been determined to be proteasome-dependent (Baldassarre et al., 2000; Urano et al., 1999). It was proposed that these negative regulatory effects of the proteasome are mediated through its role in degrading $p27^{Kip1}$and $p57^{Kip2}$. These proteins are members of the CKIs family that are responsible for withdrawal from the cell-cycle through their function in deactivating CDKs (Blagosklonny et al., 1996; Pagano et al., 1995; Sheaff et al., 2000; Sherr and Roberts, 1999; Shirane et al., 1999; Thullberg et al., 2000; Urano et al., 1999).

In contrast, the proteasomal activity was shown to stimulate cellular differentiation of a number of cell lines. For example, later stages of primary mouse keratinocyte differentiation, progression to terminal chondrogenesis, as well as RA-induced commitment of human EC NT2/D1 cell lines to differentiation are proteasome-

dependent. These effects were attributed to the proteasomal proteolysis of $p21^{WAF1/Cip1}$ or to cyclin D1 which are believed to be negative regulatory proteins for the differentiation of these cells (Di Cunto et al., 1998; Negishi et al., 2001; Spinella et al., 1999).

3) Apoptosis

Apoptosis is the process of programmed cell death that is fundamental for growth and development. Abnormalities in apoptosis contribute to a number of pathological conditions including cancers and rheumatic disorders (Thompson, 1995). Apoptosis is mainly controlled by a group of proteases called caspases, but it has also been demonstrated that the 26S proteasome plays a pivotal role in controlling apoptosis (Orlowski, 1999). More commonly, the activity of UPS was observed to be negatively correlated with apoptosis. However, a positive correlation between the proteasome and apoptosis was also identified in a number of cells such as differentiated neurons and primary cell cultures of thymocytes [reviewed in (Naujokat and Hoffmann, 2002)]. Therefore, the 26S proteasome is involved in both pro-apoptotic and anti-apoptotic functions.

A wide range of studies, using proteasome inhibitors or measuring the expression of the proteasome in carcinogenic cells have demonstrated the anti-apoptotic or survival role of the proteasome [(Drexler, 1997; Kumatori et al., 1990; Lopes et al., 1997; Qiu et al., 2000; Shinohara et al., 1996) and reviewed in (Ichihara and Tanaka, 1995)]. For example, proteasome inhibitors promote apoptosis in many malignant and rapidly growing cells including human immature leukemic cells, mouse lymphoma cells, human glioma cells, prostate carcinoma cells, proliferating bovine and human umbilical vein endothelial cells (Drexler et al., 2000; Herrmann et al., 1998; Kitagawa et al., 1999;

Kumatori et al., 1990; Naujokat et al., 2000; Tanimoto et al., 1997). Additionally, mutations within the 20S CP of yeast proteasome have shown to be associated with cell death (Heinemeyer et al., 1991).

There are various mechanisms that were proposed to explain the anti-apoptotic role of the proteasome [reviewed in (Naujokat and Hoffmann, 2002)]. These mechanisms include the role of the UPS in the proteolysis of p53, $p21^{WAF1/Cip1}$, $p27^{Kip1}$, and IκB proteins (Blagosklonny et al., 1996; Maki et al., 1996; Pagano et al., 1995; Palombella et al., 1994). These proteins are involved in regulating cell apoptosis as well as maintaining the balance between cell death and survival through their controlled synthesis, stabilization and degradation processes [reviewed in (Naujokat and Hoffmann, 2002)].

In contrast, different studies had supported the pro-apoptotic role of the proteasome in some non-proliferating or differentiating cells (Grimm et al., 1996; Sadoul et al., 1996). It has been observed that there is an increase in the abundance and proteolytic activity of the 26S proteasome during programmed cell death of Manduca sexta abdominal intersegmental muscles (Dawson et al., 1995; Jones et al., 1995; Löw et al., 1997). Furthermore, alterations in the composition of the proteasome subunits such as modifications of the ATPases within the 19S RP and addition of new subunits to the 20S RP were detected in apoptotic abdominal intersegmental muscles of Manduca sexta (Dawson et al., 1995; Löw et al., 1997). Additionally, an increase in the levels of E1, E2 and E3 enzymes, as well as an enhancement in the process of protein ubiquitination, have been reported during apoptosis of these cells (Haas et al., 1995). As well, confocal microscopy studies of immortalized cAMP-stimulated rat ovarian granulosa cells that

were undergoing programmed cell death have shown a translocation of nuclear proteasome in apoptotic blebs outside the nucleus (Pitzer et al., 1996). These findings suggest a role for the proteasome in the apoptosis of these cells.

The mechanism by which the 26S proteasome promotes a pro-apoptotic function in non-proliferating cells has not been fully investigated. Yang and colleagues in 2000 have discovered a possible mechanism that explains this pro-apoptotic role of the proteasome in primary cell cultures of thymocytes. They found that the anti-apoptotic proteins, XIAP and c-IAP1 are ubiquitinated spontaneously and degraded via the UPS when these cells are exposed to apoptotic signals (Yang et al., 2000).

4) Immunity and Cellular stress response

The peptides are the resultants of protein degradation process by the proteasome. These peptides are either used in the synthesis of new proteins or act as antigenic peptides for immunity that move to the ER and bind major histocompatibility complex (MHC) class I proteins to be presented on the surface of antigen presenting cytotoxic T lymphocytes (CTLs) (fig. 1.5 B) [reviewed in (Groettrup and Schmidtke, 1999)]. These antigenic peptides can originate from the classic 26S proteasome. However, the immunoproteasome is the major complex responsible for producing these peptides with an ideal length and perfect structure to serve as ligands for MHC class I proteins [reviewed in (Groettrup and Schmidtke, 1999)]. This complex is a specific proteasome which contains its own characterized β subunits, β1i, β2i, and β5i as well as the 11S regulatory particle [reviewed in (Wang and Maldonado, 2006)]. The assembly of immunoproteasome is promoted by interferon γ (INF-γ) signals during cellular immune response [reviewed in (Groettrup and Schmidtke, 1999)].

It had been observed that the 26S proteasome activity is up-regulated in autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus [reviewed in (Wang and Maldonado, 2006)]. Moreover, it was reported that proteasome activity correlates with the active state of these diseases. This was referred to the role of the UPS in the degradation of IκB which results in nuclear localization and hence activation of the NFκB protein. NFκB induces the formation of cytokines with their pro-inflammatory effects [reviewed in (Wang and Maldonado, 2006)]. Thus, the proteasome function is involved in both adaptive and disease-related immune responses.

Regarding the cellular stress response, the UPS is believed to participate in the response to cellular stress such as exposure to oxygen radicals, heat, heavy metals and infection (Garrido et al., 2006). This was attributed to more than one function of the UPS in regulating this response, either directly or indirectly. One of these functions is the role of UPS in targeting IκB for proteasomal degradation with subsequent activation of NFκB. NFκB induces the expression of a number of proteins including stress-response, enzymes, cell adhesion molecules and anti-apoptotic factors [reviewed in (Adams, 2003)]. Also, the unfolded heat shock proteins (e.g. chaperones, catalase) that are expressed upon accumulation of stress-induced damaged proteins are degraded via the UPS (Lee and Goldberg, 1998a). In some conditions of oxidative stress, oxidized proteins may form large aggregates that are targeted for degradation by the 20S CP without ubiquitination (Pena et al., 2007). Additionally, oxidized histones are degraded by the nuclear proteasome (Bader et al., 2007).

1.3.4 The ubiquitin/proteasome system and human diseases

Based on the involvement of the UPS in a broad range of vital cellular processes such as proliferation, differentiation and apoptosis, it is obvious that UPS is fundamental for life. Therefore, it is not surprising that malfunction or deregulation of the UPS, and the increase or decrease in the level or activity of the proteasome itself [reviewed in (Dahlmann, 2007; Naujokat and Hoffmann, 2002; Sakamoto, 2005)] are among predisposing causes of a number of acquired human diseases such as viral infections, cancers, autoimmune diseases, neurodegeneration, muscle wasting conditions, senile cataract and intolerance of old individuals to cardiac ischemia [reviewed in (Dahlmann, 2007; Naujokat and Hoffmann, 2002; Sakamoto, 2005)]. Additionally, defects in the ubiquitin/proteasome pathway play an important role in the development of genetic disorders such as cystic fibrosis, Angelman syndrome, and Liddle syndrome. Fundamentally, the impairment of ubiquitin/proteasome-mediated proteolysis participates in the pathophysiology of these diseases, either directly or indirectly, by affecting the balance between protein synthesis and degradation. Moreover, targeting the UPS is now the goal of many researchers as therapy for certain diseases [reviewed in (Sakamoto, 2005)]. In the following section, I will discuss some diseases that are associated with aberration in the UPS or related to changes in proteasome activity.

A. Genetic Disorders

1) Angelman syndrome

Angelman syndrome is characterized by a number of neurodevelopmental disorders including balance, speech, behavioral and developmental impairments. This

syndrome is a consequence of chromosomal defects on human chromosome 15q11±q13 [reviewed in (Williams et al., 2001)]. Mutations in the UbE3A gene on chromosome 15q that encodes E6-AP ubiquitin ligase protein have been reported in this syndrome. However, the precise mechanistic role underlying the function of UbE3A gene mutation in the pathogenesis of this syndrome has not been identified (Nawaz et al., 1999).

2) Liddle syndrome

This syndrome is an autosomal dominant disorder associated with hereditary hypertension due to abnormalities in water/salt balance. Several genetic disorders of β and γ epithelial Na^+ channel (ENaC) in the kidney were observed in Liddle syndrome. These genetic disorders involve either deletion of the C-terminus of β or γ ENaC or mutations in PY (Pro-Pro-x-Tyr) motif in the C-terminus of these subunits of ENaC. This motif is the binding site for E3 ubiquitin ligases. Accordingly, there are defects in the ubiquitination and degradation of β and γ ENaC which cause an increase in the number and activity of these channels at the plasma membrane and hence sodium and water retention with resultant hypertension [reviewed in (Rotin, 2008)].

B. Acquired human diseases

1) Cancers

Abnormalities in the UPS contribute to the development of several malignancies. These UPS abnormalities either enhance the uncontrolled degradation of tumor suppressors and pro-apoptotic proteins, or stabilize oncoproteins and positive cell-cycle regulators [reviewed in (Dahlmann, 2007)].

There are a number of cancers that have been attributed to aberrations in the UPS. Some cancers are attributed to the involvement of the UPS aberrations in the acceleration of pro-apoptotic and tumor suppressor proteins degradation and thus there were a decrease in the stability of these proteins or loss of function. For example, uterine cervical carcinomas initiated by the oncogenic human papilloma virus (HPV) are characterized by it's tremendously lower levels of the tumor suppressor p53. This was explained by a severe enhancement of p53 ubiquitination and proteasomal degradation through forming a complex between E6-16 or E6-18 oncogenic types of HPV, the E6-AP ubiquitin ligase enzyme and p53 (Scheffner et al., 1990). The association of p53 with E6-AP ligase was observed in these carcinomas only in the presence of the HPV oncoprotein, E6 strains. This association results in excessive imbalance between cell proliferation and apoptosis, and hence encourages neoplastic transformation (Hengstermann et al., 2001; Scheffner et al., 1990). Moreover, the CKI - $p27^{Kip1}$ - is targeted for proteasomal degradation after being attached to its specific E3 ligase, SCF_{Skp2} (Spruck et al., 2001). Increased levels of SCF_{Skp2} and consequently, accelerated proteasomal degradation of $p27^{Kip1}$ have been observed in lymphomas (Chiarle et al., 2000), gliomas (Piva et al., 1999), colorectal (Loda et al., 1997), breast (Catzavelos et al., 1997), and lung carcinomas (Catzavelos et al., 1999).

Other cancers have been attributed to a gain of function of different oncogenic and cell proliferative proteins. Abnormalities in ubiquitination enzymes or mutations in E3 ligases binding sites within protein substrates, lead to impairment of the proteolysis of oncogenic and cell proliferative proteins and thus, protein stabilization with promotion of the carcinogenesis process [reviewed in (Sakamoto, 2002)].

Multistep inherited colorectal carcinomas and malignant melanomas are examples of these cancers where the gain of function model is applied. Accumulation of the oncoprotein β-catenin was demonstrated in these tumors. β-catenin is a protein that is implicated in signaling and differentiation processes and is degraded via the ubiquitin/proteasome pathway after forming a complex with the tumor suppressor adenomatous polyposis coli (APC). Mutations in either APC or the binding site for E3 ligase within β-catenin were observed in these malignances which lead to stabilization of β-catenin and oncogenesis (Aberle et al., 1997; Inomata et al., 1996; Korinek et al., 1997; Morin et al., 1997; Powell et al., 1992; Sellin et al., 2001).

Furthermore, aberrations in ubiquitin ligases resulting in proteasome hypoactivity have been demonstrated in von Hippel-Lindau disease. This disease is a hereditary syndrome characterized by the presence of multiple cancers including retinal angiomas, cerebellar hemangioblastomas, pheochromocytoma, and renal cell carcinoma [reviewed in (Ratcliffe, 2003)]. High levels of Hypoxia-inducible factor-1α (HIF-1α) and consequently upregulation of its target genes were detected in von Hippel-Lindau disease. HIF-1α is a transcription factor that plays a pivotal role in mediating cellular response to hypoxia (low oxygen concentration) [reviewed in (Ratcliffe, 2003)]. The tumor suppressor protein, von Hippel-Lindau (VHL) is an E3 ubiquitin ligase enzyme that targets HIF-1α protein for proteasomal degradation in both normal and deficient oxygen conditions. Mutations in the second VHL allele, that cause von Hippel-Lindau disease, lead to stabilization of HIF-1α protein which in turn contributes to the hyper-vascularization or the angiogenic state of VHL disease-related tumors [reviewed in (Sakamoto, 2002)].

Finally, the implication of ubiquitin ligase impairments in the development of breast cancers that express mutant forms of human F-box protein -hCdc4- within SCF ubiquitin ligase provides more evidence that supports the role of UPS in the pathogenesis of cancers. This E3 ubiquitin ligase is responsible for targeting cyclin E for ubiquitin-mediated degradation. Accumulation of cyclin E was observed in this type of breast cancer which causes an enhancement in the proliferation of malignant cells through uncontrolled eukaryotic cell-cycle progression (Strohmaier et al., 2001).

2) Neurodegenerative diseases

It has been shown that defects in the ubiquitin/proteasome pathway are involved in the pathogenesis of neurodegeneration. Aberrations in this pathway or an age-related decrease in the activity of the 26S proteasome may be the etiologic cause or among the reasons for a number of neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), spinocerebellar ataxias (SCAs), Huntington's, Parkinson's (PD) and Alzheimer's (AD) diseases [reviewed in (Dahlmann, 2007)].

Autosomal recessive juvenile form of PD (AR-JP) is predisposed by mutations in the parkin gene within the brain stem. These mutations result in loss of the parkin protein which is an E3-ubiquitin ligase. Accordingly, the loss of parkin protein leads to failure of proteasomal degradation and therefore accumulation of numerous proteins involved in neuronal apoptosis and fated to be degraded by the 26S proteasome. Thus, neuron cells in the substantia nigra of the brain stem undergo apoptosis and AR-JP develops (Imai et al., 2001; Kitada et al., 2000; Shimura et al., 2000; Tanaka et al., 2001a). Alzheimer's disease is characterized by the presence of a ubiquitin mutant, Ub(+1) in cortical and limbic neurons. Ub(+1) competes with normal ubiquitin and impairs the process of

proteasomal protein degradation causing degeneration and cell death of neurons in the cerebral cortex and some subcortical regions and therefore AD development (Lam et al., 2000).

Additionally, the inclusion bodies that characterize several neurodegenerative diseases are composed of proteasome subunits and of ubiquitin conjugated with other proteins that are specific to each disease. For example, α-synuclein protein accumulates with ubiquitin and 19S RP within cytoplasmic inclusion bodies called Lewy bodies in idiopathic PD following the severe decrease of proteasomal proteolytic activity in dopaminergic neurons (Ghee et al., 2000; Hattori et al., 2000; Kitada et al., 2000; McNaught and Jenner, 2001; Rideout et al., 2001; Tanaka et al., 2001c; Tofaris et al., 2001). In the cerebral cortex, immunohistochemistry studies demonstrated that β-amyloid and hyperphosphorylated tau proteins are associated with both ubiquitin proteins as well as proteasome subunits within senile plaques and intracellular neurofibrillary tangles of AD respectively (Fergusson et al., 1996; Li et al., 1999). Also, the nuclear inclusions in Huntington's disease, SCAs, and Bunina bodies in ALS are other examples of neurodegenerative inclusion bodies that encompass ubiquitin and proteasomal subunits (Alves-Rodrigues et al., 1998; Chai et al., 1999; Cummings et al., 1998; Matilla et al., 2001; Sherman and Goldberg, 2001).

3) Muscle wasting conditions

Muscle wasting is associated with many pathological conditions including type I diabetes mellitus, cancer cachexia, sepsis, muscular dystrophies, chronic kidney diseases, amyotrophic lateral sclerosis, starvation, peripheral neuropathies, muscle trauma and burn injury (Attaix et al., 1998; Hasselgren et al., 2002; Kumamoto et al., 2000; Lecker et al.,

2006; Williams et al., 1999). The UPS has been observed to be linked to muscle wasting in a number of these conditions especially because of the involvement of the UPS in degrading both actin and myosin heavy chains (Ventadour and Attaix, 2006). However it is still not fully characterized whether this link is the cause or a consequence of this muscle atrophy. For example, it has been observed that the protein levels of proteasome subunits are elevated in Duchenne de Boulogne dystrophy (Kumamoto et al., 2000). Furthermore, the mRNA levels of β-subunits and α-subunits of 20S CP as well as some ATPase and some non-ATPase subunits of the 19S RP were found to be increased in muscle atrophy (Attaix et al., 1998; Combaret et al., 2005; Combaret et al., 2002; Lecker et al., 2006; Lecker et al., 2004). As well, the mRNA levels of some E3 ligase enzymes were reported to be up-regulated in the skeletal muscles of mice and rat suffering from tumor cachexia, renal failure, diabetes mellitus or during fasting (Lecker et al., 2004). Moreover, the use of proteasome inhibitors in rat with muscle wasting due to burn or sepsis results in a reduction of the muscle atrophy process which may be in part because of the inhibition of NFκB that causes catabolic muscle wasting (Cai et al., 2004; Hunter and Kandarian, 2004).

1.3.5 Proteasome inhibitors

Various proteasome inhibitors have been developed which selectively inhibit the proteolytic function of the proteasome, by blocking the active proteolytic sites within the 20S CP (Lee and Goldberg, 1998b). Proteasome inhibitors are divided into five groups according to their structures: peptide aldehydes, peptide boronates, peptide vinyl sulfones, peptide epoxyketones and β-lactones (Navon and Ciechanover, 2009). I will

introduce both peptide aldehydes e.g. N-Cbz-leu-leu-leucinal (MG132) and peptide boronates e.g. Bortezomib (fig. 1.6) (Zhu et al., 2005).

Peptide aldehydes are synthetic, potent, permeable and reversible inhibitors. Some members of this group excluding MG132 have an inhibitory effect toward other cellular proteases and calpains. The inhibitors of this group selectively block the peptidase activities of both chymotryptic and post-acidic subunits of the 20S CP [reviewed in (Adams, 2003)]. Peptide aldehydes are the most widely used group in research fields as they did not impair cell growth and survival for 10-20 hours (Lee and Goldberg, 1998b). In this study I used MG132 as a member from this group.

MG132 is used broadly in cancer research and its use helped to discover that inhibition of the 26S proteasome function increases the sensitivity of many cancer cells to radiotherapy and chemotherapy. For example, MG132 pretreatment increases the sensitivity of radioresistant HG-My-Z Hodgkin's lymphoma cells to radiation as well as of pancreatic cancer cells to etoposide and doxorubicin chemotherapies (Arlt et al., 2001; Frank et al., 2000).

Peptide boronates are the most potent proteasome inhibitors. They are reversible, highly penetrable to the cells and specific to the proteasome without affecting other cellular proteases. Peptide boronates selectively block chymotryptic-like peptidase activity of the 20S CP. In this study, we used the dipeptide boronate, bortezomib (formerly named PS-341, LDP-341, and MLNM341) (Richardson et al., 2003; Zhu et al., 2005).

Bortezomib is more potent, more stable, and more specific to the proteasome than MG132 [reviewed in (Adams, 2003)]. This agent is the first proteasome inhibitor to be

used in clinical trials for cancer treatment as it causes apoptosis of tumor cells with no significant toxic effects on non-carcinogenic cells (Fernández et al., 2006). Bortezomib (also known as Velcade) is now approved by the FDA as a third-line chemotherapeutic agent for treating relapsed and refractory multiple myeloma cancer patients [(Anderson, 2004); http://www.fda.gov/Drugs/NewsEvents/UCM130961]. Multiple myeloma is cancer of blood origin from the plasma cells. This cancer is the second most common hematological malignancy that resulting in about 2% of all cancers mortality (Collins, 2005). The mechanism of action that underlies the effect of bortezomib on multiple myeloma cells is either directly on myeloma cells or by affecting the bone marrow tissue where the tumor grows and so inhibiting the tumor proliferation. Bortezomib acts on myeloma cells through its effect on stabilizing IκB that blocks the cellular survival protein NFκB which promotes the transcriptional activation of several genes involved in cell-cycle regulation, cell adhesion, and anti-apoptotic function. By blocking this factor the growth and proliferation of myeloma cancer cells will be repressed. Moreover, NFκB helps in adhering the myeloma cells to the bone marrow and thus stimulates carcinogenesis which again will be abolished by blocking this factor (Anderson, 2004).

Studies using bortezomib as a single therapy in treating ovarian cancers, and head and neck squamous cell carcinomas have confirmed the cytotoxic or antitumor effects of this agent (Frankel et al., 2000; Sunwoo et al., 2001). Additionally, Teicher *et al.*, demonstrate that using bortezomib, either alone or in conjunction with other chemotherapeutics decreases the process of metastases in a mouse model for lung metastasis (Teicher et al., 1999). Also, it has been reported that bortezomib increases the sensitivity of prostate cancer xenografts to radiotherapy (Pervan et al., 2001), of

pancreatic cancer xenografts to chemotherapy (Richard et al., 2001; Shah et al., 2001) and of colon tumor xenografts to both radiation and chemotherapy (Cusack et al., 2001; Suzanne et al., 2001). Several molecular mechanisms for the mode of action of bortezomib in enhancing the response of tumor cells to chemotherapy may be implicated beside its role in blocking NFκB. These include induction of p21, p27 and p53 that were demonstrated after proteasomal inhibition in colon tumor xenografts treated with chemotherapy (Cusack et al., 2001).

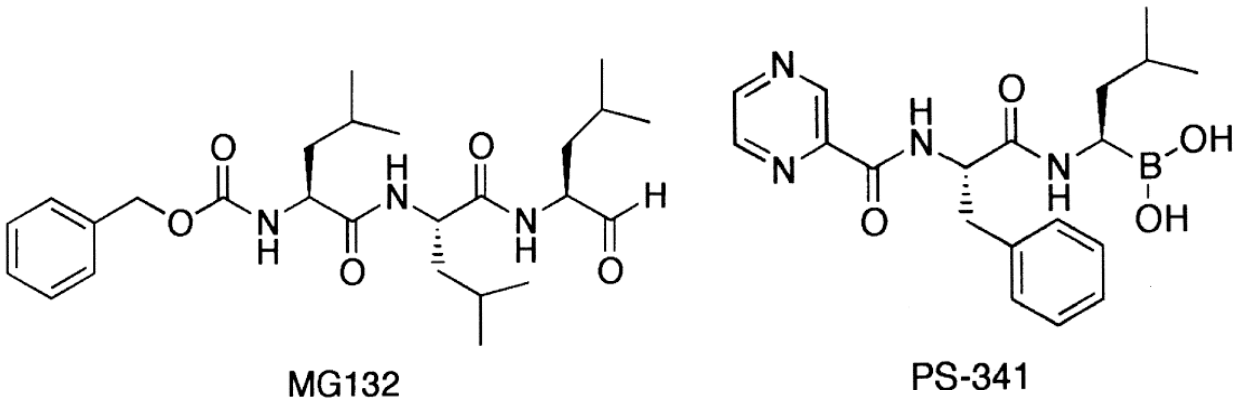


Figure 1.6 **Chemical structures of MG132 and bortezomib**

Diagramatic illustration of the proteasome inhibitors used in this study. MG132 (left) is a peptide aldehyde proteasome inhibitor whereas PS-341 (right) is a peptide boronate proteasome inhibitor.

1.4 Posttranslational modifications

Proteins are subjected to different modifications after their translation in processes called post translational modifications (PTMs). In these processes, chemical groups such as acetyl, phosphate, and methyl groups or small proteins including ubiquitin and Sumo proteins are added to the amino acids within these proteins (Seo and Lee, 2004; Tini et al., 2009). Post translational modifications of proteins regulate their functions and accordingly their dependent biological processes and signaling pathways through modulating their activities, subcellular localization and their association with other proteins (Seo and Lee, 2004; Tini et al., 2009). Also, these protein modifications may cause changes in the tertiary structures of proteins which assists in creating their multi-functionality (Seo and Lee, 2004). During my Ph.D. studies, I investigated the ubiquitination of retinoid receptors. Indeed, this post translational modification is known to affect the expression of different genes including retinoic acid-responsive genes.

1.4.1 Ubiquitination

The ubiquitin is a highly conserved 76 amino acid protein with a molecular weight of about 8.6 kDa (Weissman, 2001). Ubiquitin binds to internal lysine residues within protein substrates (Ciechanover and Ben-Saadon, 2004). As previously mentioned, the process of protein ubiquitination is linked usually to proteasome degradation. However, it has been shown that protein ubiquitination plays a critical role in regulating gene transcription as well (Muratani and Tansey, 2003). Furthermore, protein ubiquitination was demonstrated to affect protein localization, activity, stability and binding to other proteins (Balastik et al., 2008). The polyubiquitination process which was thought to be

the hallmark of protein degradation via the 26S proteasome has been reported to be involved in determining the fate of many protein substrates, which may not be proteolysis, depending on the characters of this polyubiquitination. If the ubiquitin proteins within the polyubiquitin chain bind to each other through lysine 48 (K48), the protein will be degraded by the 26S proteasome. However, if the target protein is bound to a polyubiquitin chain that has ubiquitin molecules attached to each other through Lysine 63 (K63) or have less than four ubiquitins in the chain, the substrate will not undergo proteolysis (Muratani and Tansey, 2003). Therefore, it appears that ubiquitination is involved in other functions, in addition to protein degradation (Muratani and Tansey, 2003).

1.4.2 Role of ubiquitin/proteasome system in gene transcription control

The ubiquitination process is involved in regulating gene transcription either through its role in protein proteolysis via the proteasome, or through proteasome-independent non proteolytic function. Interestingly, these two apparently opposite functions are in fact linked together to regulate gene transcription (Conaway et al., 2002).

The proteasome-independent function of ubiquitination in regulating gene expression is mainly linked to its role in the control of chromatin by ubiquitinating core histones (Muratani and Tansey, 2003; Ulrich, 2002; Zhang, 2003). In order to understand this section, I will describe briefly the structure of nucleosomes which are the basic units of eukaryotic chromosomes (fig. 1.7). The eukaryotic DNA is packed into the nucleus by wrapping tightly around nucleosomes (fig. 1.7). The nucleosome contains about 150 base pairs of DNA wrapped around the nucleosome core in 1.75 turns of left-handed superhelical DNA (fig. 1.7). This core is formed from histones termed core histones

which include histones H3, H4, H2A and H2B. The nucleosome is composed of a histone octamer that has two H3, two H4, two H2A and two H2B histones. In contrast, the H1 histone functions as a linker histone that connects neighboring nucleosomes (fig. 1.7) (Kornberg, 1974; Luger et al., 1997). Histone surfaces bind to DNA by H-bonds and electrostatic interactions (Bottomley, 2004).

Histone H2A was found to be ubiquitinated, and this ubiquitination is associated with both active chromatin as well as inactive transcriptional regions (Baarends et al., 1999; Barsoum et al., 1982; Levinger and Varshavsky, 1982; Nickel and Davie, 1989). Furthermore, histone H3 has been demonstrated to be ubiquitinated in elongating spermatids (Chen et al., 1998). The yeast H2B core histone is ubiquitinated by E2 conjugating enzyme, Rad6 at lysine 123 (K123) which leads to methylation of H3 at lysine 4 and 79 (K4, 79). This histone H3 methylation results in telomeric gene silencing (Briggs et al., 2002; Davie and Murphy, 1990; Huang et al., 1997; Robzyk et al., 2000; Sun and Allis, 2002). Additionally, silencing of the ARG1 gene that encodes argininosuccinate synthetase is caused by histone H2B ubiquitination via Rad6 (Turner et al., 2002). In contrast, ubiquitinated histone H2B is required transiently for SAGA (Spt-Ada-Gcn5-acetyltransferase)-dependent gene transactivation (Henry et al., 2003). The TATA binding protein "TBP" associated factor 1 (TAF 1) causes ubiquitination of linker H1 histones which leads to transcriptional activation (Pham and Sauer, 2000).

Another mechanism underlying the role of ubiquitination in transcription is its effects on regulating the level, localization and activity of transcription factors and their coregulators (Muratani and Tansey, 2003). These effects are due to the function of ubiquitination in proteasome-dependent proteolysis of these proteins. This function

coordinates transcription by providing the cell with the correct proteins, at the right place, with the right activity and at the right time. Examples of this role include: 1) the effects of UPS in activating NFκB (Palombella et al., 1994), 2) the role of UPS in regulating the exchange between corepressors and coactivators (Collins and Tansey, 2006), 3) The function of UPS in preserving the low levels and instability of β-catenin, p53 as well as HIF-1α in non induced cells which could be activated with specific signals (Aberle et al., 1997; Haupt et al., 1997; Maxwell et al., 1999), and 4) The UPS mediated transcription coupled destruction of SMAD2 to limit its uncontrolled activity (Lo and Massague, 1999).

Finally, the 19S RP of the 26S proteasome was shown to be involved in gene transcription (Gonzalez et al., 2002; Muratani and Tansey, 2003). SUG-1 and SUG-2 components of the 19S RP are involved in the elongation steps of transcription in association with RNA Pol II (Ferdous et al., 2002). Moreover, the SUG-1 subunit has been shown to interact with TBP, TFIIH and RNA Pol II (Makino et al., 1999; Melcher and Johnston, 1995; Weeda et al., 1997). Indeed, deletion of SUG-1 leads to inhibition of RNA Pol II- mediated transcription (Makino et al., 1999; Sun et al., 2002). In line with this theme, the ATPase subunits of the 19S RP appear to play a role in modifying core histones as well as transcriptional activator complexes such as the histone acetyltransferase SAGA (Spt-Ada-Gcn5-acetyltransferase) complex (Ezhkova and Tansey, 2004; Lee et al., 2005). Additionally, the UPS is implicated in the process of transcription-coupled repair (TCR) via RNA Pol II. Upon DNA damage, elongating RNA Pol II covers the damaged DNA region. This RNA Pol II is then subjected to phosphorylation at its C-terminus. This phosphorylation triggers Pol II ubiquitination via

ubiquitin ligase, Rsp5 which is followed by Pol II proteasomal destruction and recruitment of DNA repair machinery (Beaudenon et al., 1999; Huibregtse et al., 1997; Lee et al., 2002; Muratani and Tansey, 2003).

1.4.3 Epigenetic crosstalk between the ubiquitin/proteasome system and RAR-regulated genes transcription

A number of studies in the last ten years had shown that the activity of the UPS is vital for activating RAR/RXR mediated genes transcription (Bastien and Rochette-Egly, 2004; Gianni et al., 2002; Gianni et al., 2006; Perissi et al., 2004; Perissi et al., 2008). Additionally, it has been reported that retinoid receptors as well as their coactivators are degraded via the UPS upon ligand binding (Boudjelal et al., 2000; DeMartino and Slaughter, 1999; Gianni et al., 2002; Gianní et al., 2003; Kopf et al., 2000; Tanaka et al., 2001b; Zhu et al., 1999a). However, there is a contradiction between ligand-dependent degradation and transactivation. Different explanations have been proposed to solve this conflict. These explanations suggest that the ligand-dependent proteasomal degradation is critical for limiting the magnitude and length of RA-responsive gene transactivation and hence participates in preventing uncontrolled activation of these genes (Bour et al., 2007). Additionally, other explanations propose that this degradation is required for cyclic recruitment of retinoid receptors/activators complexes (Bour et al., 2007). Therefore, in the presence of ligand, fresh transcriptional complexes will be provided to RA target promoters and many cycles of transcriptional induction will be conducted (Bour et al., 2007). Moreover, the UPS is required to clear out retinoid receptors corepressors which have been demonstrated to be targets for proteasomal degradation.

As a result, free space for coactivators binding will be available (Collins and Tansey, 2006; Dennis and O'Malley, 2005; Lipford and Deshaies, 2003; Perissi et al., 2004; Perissi et al., 2008).

In addition, there is an increasing body of evidence suggesting a connection between the non-proteolytic functions of the proteasome and the transcriptional activity of retinoid receptors. This evidence includes the association between SUG-1 subunit (one of the six ATPases in the 19S RP of the 26S proteasome) and the AF-2 region of liganded RAR-γ2 following a series of phosphorylation and ubiquitination of the AF-1 region of this receptor. This association was found to be fundamental for transcriptional activation mediated by RAR-γ2/RXR heterodimers (Gianni et al., 2002; vom Baur et al., 1996). Also, it has been demonstrated that SUG-1 subunit of the 19S proteasome is recruited to the AF-2 region within liganded RAR-α in association with steroid receptor coactivator 3 (SRC-3) and hence, activates the transcription of RAR-α mediated genes (Ferry et al., 2009). Finally, it has been revealed that E3 ubiquitin ligase - TBLR1 (Transducin β-like protein 1-related protein) - binds to retinoid receptors and functions as coregulator for RAR-mediated transactivation. Upon ligand induction, this E3 ligase component are recruited and bound to UbcH5 or UbcH7 or E6-AP or MDM2/19S proteasome. This facilitates coactivators binding to retinoid receptors (Perissi et al., 2004; Perissi et al., 2008).

Collectively, these evidences strongly suggest that the involvement of the UPS in activating RA-responsive genes is a highly complex process that includes both proteolytic and non-proteolytic activities of the proteasome.

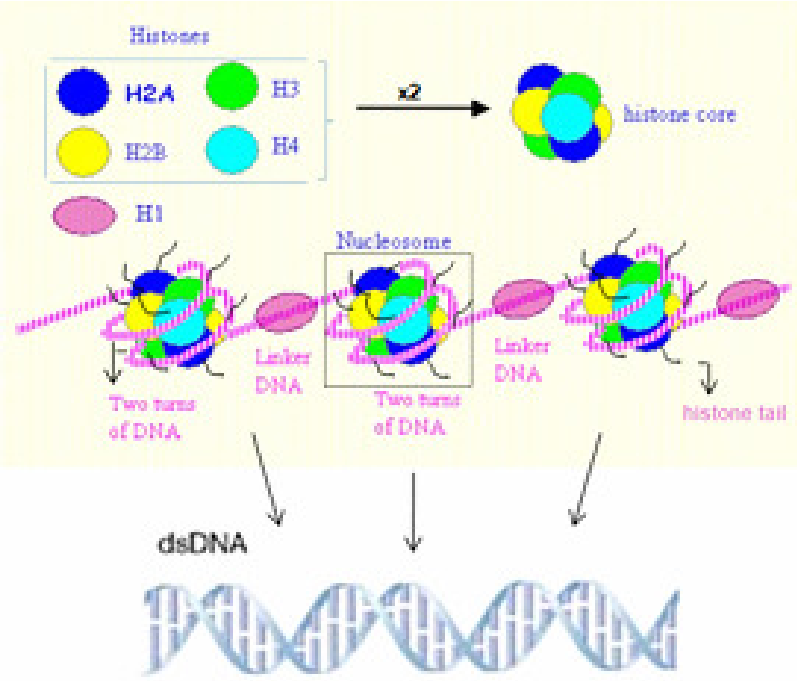


Figure 1.7 **Structure of the nucleosome as a DNA packing unit**

Adapted from http://staff.um.edu.mt/acus1/6Cromosomes.htm and (Bottomley, 2004)

DNA is packed into the nucleus in the form of chromatin. Chromatin is formed by many nucleosomes. Each nucleosome is composed of a core of histones comprising a histone octamer from 2 H3, 2 H4, 2 H2A and 2 H2B histones around which about 2 turns (1.75 turns) of DNA are wrapped. Neighboring nucleosomes are connected together by linker DNA (10-80 base pairs) and linker histone H1.

CHAPTER 2: RATIONALE, OBJECTIVES AND HYPOTHESIS

2.1 Rationale

The transcription of RA-responsive genes is controlled in a ligand-dependent manner through the exchange between co-regulatory protein complexes that consist of coactivators and corepressors (Xu et al., 1999a). During the last ten years, a number of studies had demonstrated that RA-responsive genes are also regulated via unconventional coregulators such as the proteasome and posttranslational modifications which affect retinoid receptors (Bour et al., 2007; De los Santos et al., 2007; Gaillard et al., 2006; Perissi et al., 2004).

In the current study, we focused on the fundamental role of the ubiquitin/proteasome pathway as an activator for RAR-regulated genes. It has been reported that the activity of the 26S proteasome is crucial for RA-responsive gene transactivation (Perissi et al., 2004). However, the exact role of the ubiquitin/proteasome system (UPS) in regulating these genes as well as the mechanisms that trigger this transactivation were not identified (Bastien and Rochette-Egly, 2004). In addition, a number of studies had shown that the subcellular localization of retinoid receptors is not restricted to the nucleus as previously thought but their cytoplasmic localization is also possible (Akmal et al., 1998; Dufour and Kim, 1999; Ghose et al., 2004; Tahayato et al., 1993; Weis et al., 1994; Wu et al., 2004). Different regulators for the nuclear/cytoplasmic trafficking of retinoid receptors have been suggested including RA and protein kinase C (Akmal et al., 1998; Tahayato et al., 1993; Weis et al., 1994). Importantly, these regulators as well as subcellular localization of retinoid receptors are

cell type- and retinoid receptor subtype-specific (Dufour and Kim, 1999). Therefore, further investigations are needed to identify other regulators of retinoid receptors intracellular trafficking in different cell lines. Moreover, it was demonstrated that retinoid receptors are ubiquitinated in response to ligand induction (Boudjelal et al., 2000; DeMartino and Slaughter, 1999; Gianni et al., 2002; Gianní et al., 2003; Kopf et al., 2000; Tanaka et al., 2001b; Zhu et al., 1999a) but whether this ubiquitination is only necessary for retinoid receptors destruction or also plays a role in their homeostasis is still not clear.

2.2 Overall objective

Our study was designed to decipher the molecular mechanisms underlying the involvement of the ubiquitin/proteasome system in the regulation of RAR-α/RXR-α-dependent genes in P19 cells, particularly upon induction with a RAR-specific ligand. Understanding the molecular basis of such involvement may provide novel insights into the role of these unconventional coregulators in the dynamics of RAR-dependent transcriptional activity.

2.3 Hypothesis

We postulated that the non-proteolytic function of the UPS is fundamental for maintaining the occupancy of RAREs in the vicinity of TATA box by liganded retinoid receptors/coactivators complexes.

2.4 Specific objectives

1. To investigate the role of the 26S proteasome in the occupancy of RARE by RAR-α/RXR-α, their coregulators as well as RNA polymerase II.

2. To examine the effect of proteasome inhibition on the DNA-independent association between RAR-α and its cofactors.

3. To detect the consequences of RAR-specific ligand and proteasome inhibition on the subcellular distribution of retinoid receptors and their coactivators in P19 cells.

4. To reveal the role of RAR-specific ligand activation on the ubiquitination and stability of retinoid receptors.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell culture and Reagents

P19 cells were kindly provided by Dr. Jim Dimitrolakos and Dr. Ilona Skerjanc. The cells were grown in tissue culture dishes or flasks and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% non-essential amino acids at 37ºC with 5% CO2. The cells were split every other day and treated after settlement on the tissue culture dishes for at least 16 hours. TTNPB (1 µM) ± MG132 (5 µM) or bortezomib (5, 10, 20, or 40 nM) were used for treating cells. Cycloheximide (CHX 10 µg/ml) was used to stop the synthesis of RXR-α and hence to assist in measuring its half life. The media was changed each time before starting the treatment. All the reagents and drugs that were used in this project are mentioned in appendix A "Table 1".

3.2 Cell transfection and Luciferase assay

The cells were cultured in 6-well plates as mentioned above in "Cell culture" for at least 16 hours. Following that, transient transfection was performed for 4-6 hours at 37 ºC using 1.2 µl per transfection of the polyethylenimine agent ExGen 500 mixed with 0.3 µg per transfection of RAR-β2-responsive reporter DNA (βRE3-Luc) or 0.3 µg per transfection of respiratory syncytial virus β-Galactosidase (RSV-β-Gal), a β-Galactosidase vector which functions as a reference plasmid for internal control mixed with carrier DNA. The transfection was stopped by removing the old media and adding new DMEM supplemented with 10% FBS to the cells then treating them as indicated in

fig. 4.1 A. At the end of the indicated treatment times, cells were washed, harvested, lysed and extracts were assayed for luciferase and β-Galactosidase activities using Promega luciferase assay system Kit according to the manufacturer's instructions. The luciferase and β-Galactosidase activities were measured utilizing a micro plate luminometer and the readings of luciferase activities were normalized to β-Galactosidase activities and expressed as fold induction relative to untreated controls.

The reporter used in this assay is mentioned in appendix B "Table 1". The kit used for measuring luciferase and β-Galactosidase activities is mentioned in appendix A "Table 1".

3.3 Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR)

P19 cells were cultured in 6 wells plates as described before in "Cell culture" and then treated. Total RNA from cultured cells was extracted using either Total RNA Kit I or RNeasy Mini Kit according to the manufacturer's instructions. In brief, a lysis buffer containing guanidine thiocyanate and β-mercaptoethanol was used to homogenize these cells. This is followed by addition of 70% ethanol to maximize the binding of the extracted RNA to the silica-gel spin columns. After that, the homogenized cells and ethanol mixes were spun through RNeasy Mini spin columns. The RNA then remained bound to the columns and was subjected to several washes to remove out any contaminants. Next, RNase-free water was used to elute RNA from the columns. Finally, the concentrations of the extracted RNA were measured using a Multiscan Spectrum Photospectrometer (Thermo scientific, Lafayette, CO, USA). The relative

purity was identified by the ratio of the readings at 260 nm and 280 nm absorbance. Reverse transcription was then performed with 2 µg of RNA per reaction using High Capacity cDNA Archive Kit as described in manufacturer's protocol. The cDNA from the reverse cross linking step was used in Quantitative real time RT-PCR analysis using Power SYBR® Green PCR Master mix plus gene-specific primers (RAR-β2 and Cyp26A1) and utilizing the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control to normalize the expression of target genes and quantify their mRNA levels.

PCR conditions following activation for 10 minutes at 95°C were repeated for 40 cycles as the following: denaturation (95°C) for 15 seconds, annealing (primers-specific temperature) for 30 seconds, extension (72°C) for 35 seconds. Results are analyzed by cycle threshold (Ct) comparative method where the $2^{-\Delta\Delta Ct}$ values were calculated ($\Delta Ct = Ct_{target\ gene} - Ct_{GAPDH}$ & $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$). The reagents and primers that were used are listed in appendix A "Table 1" and appendix B "Table 2" respectively.

3.4 Whole cell extracts and Western blot analysis

Cells were maintained as described earlier in "Cell culture", treated then washed 2 times with Phosphate-Buffered Saline (PBS) and harvested. The harvested cells were pelleted by centrifugation at 3,000 rpm for 3 minutes at 4˚C. The Whole cell extracts were prepared by incubating the cell pellets in whole cell extract buffer (10% glycerol, 50 mM Tris-HCl pH 7.6, 400 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF and 1% NP-40) for 30 minutes on ice followed by centrifugation (14,000 rpm) for 15 minutes at 4˚C. Bradford assay was used to measure the protein concentrations in whole cell extracts by Bio-Rad Protein Assay Dye Reagent and Multiscan Spectrum

Photospectrometer (Thermo scientific, Lafayette, CO, USA). Equal amounts of protein (50 µg) were diluted in 2X Laemmli buffer (25% glycerol, 125 mM Tris-HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 0.01% bromophenol blue), heated at 95^{o}C for 5 minutes and finally loaded and resolved by SDS polyacrylamide gel (6% or 8%). Following that, the proteins were transferred to or blotted onto Immun-Blot PVDF membranes either for 1 hour at 100 Volt or overnight at 35 Volt according to the protein of interest. Upon transfer, the membranes were blocked with PBST (1% Tween in PBS) supplemented with 5% non-fat milk for 1 hour at room temperature. The membranes were then incubated overnight with the corresponding primary antibodies diluted in PBST/1% milk at 4°C. Next, the membranes were washed 3 times for 5 minutes each with PBST. Primary antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies raised against the corresponding species diluted in PBST/1% milk for 1 hour at room temperature followed by washing again with PBST for 1.5 hour totally (3 times). Finally, peroxidase activity was visualized with Western Lightening ™ Enhanced Chemiluminescence (ECL) solution using X-ray film developer machine. Signal intensity was quantified densitometrically using Scion Image software, version 4.02 (Scion Corporation, Frederick, MD, USA). Even loading between lanes was determined by the densitometry of γ tubulin blots.

The reprobing of the membranes was performed after stripping the membranes at 50°C for 30 minutes using membranes stripping buffer (2% SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β-mercaptoethanol). The membranes were then blocked with 5% milk in PBST for 1 hour and reprobed with the primary and secondary antibodies as described above.

The primary antibodies that were used for Western Blots in this project are anti RAR-β2 (C19) in 1:500 dilution, anti RAR-α (C20) in 1:500 dilution, anti RXR-α (D20) in 1:500 dilution, anti p300 (N15) in 1:500 dilution, anti SRC-1 (M-341) in 1:500 dilution, anti N-CoR (C20) in 1:500 dilution and anti γ tubulin (GTU-88) in 1:10000 dilution. The secondary antibodies are HRP-conjugated goat anti-rabbit IgG (1:10000), goat anti-mouse IgG (1:40000) and donkey anti-goat IgG (1:40000) secondary antibodies (appendix A "Table 2"). The reagents that were used in this assay with their suppliers are mentioned in appendix A "Table 1".

3.5 Immunoprecipitation (IP) and Coimmunoprecipitation assays

Cultured cells were treated for 4 hours with TTNPB (1 μM) and/or MG132 (5 μM) and then the whole cell extracts were collected and analyzed for total protein content as described above in "Western blot". Equal amounts of protein extracts were diluted with IP dilution buffer (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF and 1 mg/ml BSA) and incubated overnight with antibodies specific to the protein of interest (2 μg/reaction) at 4˚C with gentle rocking. After that, protein A-agarose beads were added to the mix for 2 hours at 4˚C and the agarose beads were pulled down by centrifugation at 3,000 rpm for 1 minute. Then the precipitates were washed 3 times by PBS with 1% Triton X-100. After the final wash, 2X Laemmli buffer was added to the immunoprecipitates and heated for 5 minutes at 70˚C. The heated samples were vortexed for 20 seconds, centrifuged at 3,000 rpm for 1 minute, heated again as before then loaded and separated onto SDS-PAGE. Finally, the proteins were transferred to the membranes and detected using "Western blot" as described earlier with the same procedures for membranes reprobing.

The antibodies that were used for IP are anti RXR-α (D20), anti SRC-1 (MA1-840), anti p300 (Ab-1) and anti N-CoR (C20). The antibodies against RAR-α (C20) and RAR-α (MA1-810) were used for Western Blotting assays with the same dilutions as mentioned before to detect the coimmunoprecipitated proteins. For the *in vivo* ubiquitination assay, IPs were performed using anti RAR-α (C20) and anti RXR-α (D20). Western Blots were done using Ub (P4D1) in a dilution of 1:200 (appendix A "Table 2").

3.6 Chromatin Immunoprecipitation (ChIP) assay

After treatment, the cells were cross-linked with 1% formaldehyde in DMEM for 15 minutes at 37˚C. Next, the fixation was quenched by 200 mM Glycine. The fixed cells were washed for 2 times with ice-cold PBS, harvested and pelleted as described earlier in Western blot assay. The cells were then lysed for 10 minutes on ice using ChIP lyses buffer (1% SDS, 50 mM Tris-HCl pH 8.0, 10 mM EDTA and 1 µl/ml protease inhibitors cocktail). Lysed cells were sonicated for 14 minutes using Bioruptor system (Diagenode, Denville, NJ, USA) with 30 seconds on/off cycle at high setting. Sonicates were centrifuged at 14,000 rpm for 15 minutes and the supernatants were pre-cleaned for at least 2 hours at 4˚C by Protein A-agarose/Salmon sperm DNA beads blocked with IgG free BSA. Upon pre-cleaning, DNA concentrations were measured and 2.5% of the lysate were removed to be used as input controls. Equal amount of DNA were diluted at least 10 times in ChIP dilution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 1µl/ml protease inhibitors cocktail) and Chromatin immunoprecipitation was performed using 2 µg/reaction of antibodies against the target proteins for overnight incubation at 4˚C. Protein A-agarose/Salmon sperm DNA were then added to the mix for at least 2 hours at 4˚C to pull down the chromatin/antibodies

complexes. The immune complexes were then washed sequentially for 10 minutes each with ChIP washing buffer A (0.1% SDS, 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA pH 8.0 and 1% Triton X-100), buffer B (0.1% SDS, 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM EDTA pH 8.0 and 1% Triton X-100), buffer C (1% sodium deoxycholate, 20 mM Tris-HCl pH 8, 0.25 M LiCl, 1 mM EDTA pH 8.0 and 1% NP-40) and 2 times with TE buffer (10 mM Tris-HCl pH 8.0, and 1 mM EDTA pH 8.0). Immune complexes were then eluted from the beads with elution buffer (1% SDS and 0.1 M $NaHCO_3$) by agitation for at least 15 minutes at room temperature. Next, reverse cross linking were performed overnight at 65˚C using proteinase K enzyme. The immunoprecipitated DNA fragments were purified using Cycle Pure PCR purification Kit and amplified by PCR reactions using equal amounts of elutes, specific primers, dNTP mix, and Go Taq® flexi DNA polymerase PCR kit. The products of PCR were then resolved on 2% agarose gel stained with ethidium bromide. The AlphaImager ™ (Alpha Innotech, San Leandro, CA, USA) was used to visualize DNA bands.

The DNA bands were quantified using Scion Image software (Scion Corporation, Frederick, MD, USA) after being normalized to input controls. The antibodies used for ChIP are anti RAR-α (C20), anti RXR-α (D20), anti p300 (C20), anti SRC-1 (M-341), anti N-CoR (C20) and anti Pol II (A-10) (appendix A "Table 2"). The primers used in this approach are mentioned in appendix B "Table 3".

3.7 Immunofluorescent staining

P19 cells were grown on cover-slips overnight and treated thereafter for 4 hours with TTNPB (1 μM) and/or MG132 (5 μM) in comparison to untreated cells. After treatment, cells were washed twice with PBS and fixed with 4% freshly prepared cold paraformaldehyde for 15 minutes on ice. The fixed cells were permeabilized with 0.2% NP-40 for 15 minutes at room temperature and blocked with PBST/milk (1X PBS, 10% milk, 0,2% Tween 20) for 5 minutes at room temperature. The cover-slips were then incubated with specific primary antibodies diluted in PBST/milk for overnight at 4˚C followed by 5 times washing with PBS. Next, the cells were incubated for 30 minutes with specific PBST/milk diluted secondary antibodies at room temperature in total darkness. When indicated, cells may be washed with PBS again and incubated with 0.025μg/ml Hoechst for 10 minutes in darkness at room temperature. Finally, the cover-slips were mounted with 90% glycerol before sealing. The fluorescent signals were visualized by utilizing 63x Plan Apochromat oil-immersion objective (Carl Zeiss Inc., Germany) of immunofluorescent microscopy with ApoTome optical sectioning (Axiovert 200M microscope "Zeiss", Germany), AxioCam HRM camera (Zeiss, Germany) and AxioVision Rel 4.6 software (Zeiss, Germany). The Z optical sectioning was performed at an optical depth of 0.25 μm. Channel images were merged using Adobe Photoshop 7.01 (Adobe, Ottawa, Canada).

All the antibodies (either primary or secondary) used for immunofluorescent staining were in 1:200 dilutions. The primary antibodies that were used in the current study include anti RAR-α (C20), anti p300 (N-15), anti Ub (P4D1) and anti α-subunits of

the 20S proteasome α7/α8 (B-4) (appendix A "Table 2"). The used reagents are mentioned in appendix A "Table 1"

3.8 Pulse Chase assay

Cells were cultured at least 24 hours before starting the experiment, washed with PBS and then incubated with methionine/cysteine free DMEM media (appendix A "Table 1") for at least 30 minutes at 37˚C with 5% CO2. The Pulse phase was then started by labeling the cells with 10 µCi/ml of ^{35}S-methionine/cysteine (appendix A "Table 1") for 4 hours at 37˚C. Following that, the cells were washed with PBS for 3 times and then the Chase phase was conducted by adding regular DMEM to the cells and starting their ligand induction for the indicated time points after harvesting the control (without chase plates). At the indicated time points, the cells were washed and harvested. Immunoprecipitation and Western blotting were then performed as described earlier using anti RAR-α (C20) antibody (appendix A "Table 2") for IP except that there was no transfer to the membrane. Instead the gel was fixed after electrophoresis, amplified according to manufacture recommendations (Perkin Elmer, Woodbridge, ON, Canada) and finally left overnight to dry. Next, the gel was scanned with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA, USA) and the bands were quantified by Scion Image software (Scion Corporation, Frederick, MD, USA) to determine the half life of the target protein from the remaining radioactivity of the immunoprecipitated proteins after chase.

3.9 Statistical analysis

Results are represented as mean ± S.D. Statistical analysis was carried out by Student's *t*-test. Statistical significance was described by $p\ value < 0.05$ and highly significant by $p\ value < 0.005$.

CHAPTER 4: RESULTS

4.1 The 26S proteasome function is essential for transactivating the RAR-regulated gene RAR-β2 in P19 cells

It was established that the function of the 26S proteasome plays a pivotal role in the activation of RA-responsive genes (Bastien and Rochette-Egly, 2004; Gianni et al., 2002; Gianni et al., 2006; Perissi et al., 2004; Perissi et al., 2008) whereas the underlying mechanisms of this role is still not fully understood. In the current study, we wanted to recognize the mechanistic role of the proteasome on the expression of RAR-regulated genes. We used the P19 cell line to achieve our goals as these cells showed an early induction of many RA-responsive genes including RAR-β2 and Cyp26A1 (Vucetic et al., 2008). Furthermore, we used the RAR-specific ligand TTNPB to induce our target genes as we were interested in demonstrating the specific function of RAR-α in this scenario.

Initially, we studied the effects of the proteasome on RAR-β2 gene transactivation which is mediated by ligand induction of RAR/RXR heterodimers through binding to β2-RARE (Pratt et al., 2000). First, the requirement of the 26S proteasome for RARE-mediated transactivation was identified via luciferase assay. P19 cells were transfected with β2-RE reporters and then induced with 1 μM of RAR-selective ligand TTNPB in the presence or absence of 5 μM of the proteasome inhibitor MG132 for 16 hours. After that, luciferase activities were measured in these cells in comparison to untreated controls. We found that cells treated with RAR-specific ligand TTNPB showed a large induction of luciferase activity (about 50 fold) in comparison to untreated cells or cells

treated only with proteasome inhibitor MG132. This induction was severely inhibited after inhibition of the 26S proteasome by MG132 in ligand-induced cells (fig. 4.1 A).

Next, we demonstrated the effects of the 26S proteasome activity on the expression of the endogenous RAR-β2 gene. P19 cells were treated with RAR-specific ligand TTNPB and/or proteasome inhibitor MG132 for 1, 2, 3, 4 and 16 hours then RAR-β2 mRNA levels were measured using real-time RT-PCR. We found that RAR-β2 mRNA levels were decreased in a time dependent manner in cells treated concomitantly with MG132 and TTNPB in comparison to cells treated only with TTNPB (fig. 4.1 B). This decrease was observed from 2 hours and reached to more than 50% after 4 hours of concomitant treatment. Finally, the effects of proteasome inhibition on RAR-β2 protein levels were detected. Quantitative Western blot assays were done using equal amounts (50 µg) of P19 cells whole cell lysates induced with RAR-specific ligand TTNPB in the presence or absence of the proteasome inhibitor MG132 for 4, 8 and 16 hours in comparison to untreated control cells. It was shown that proteasome inhibition had reduced RAR-β2 protein levels in TTNPB induced cells (lanes 4, 7 and 10) starting from 4 hours of cotreatment in comparison to TTNPB induced only cells (lanes 3, 6 and 9) (fig. 4.1 C & D). Moreover, RAR-β protein levels were severely low in untreated control cells (lane 1) or MG132 treated only cells (lanes 2, 5 and 8).

All together, these data indicate that the function of the 26S proteasome is essential for RARE-dependent RAR-β2 gene transactivation in P19 cells. This transactivation is mediated by liganded endogenous RAR.

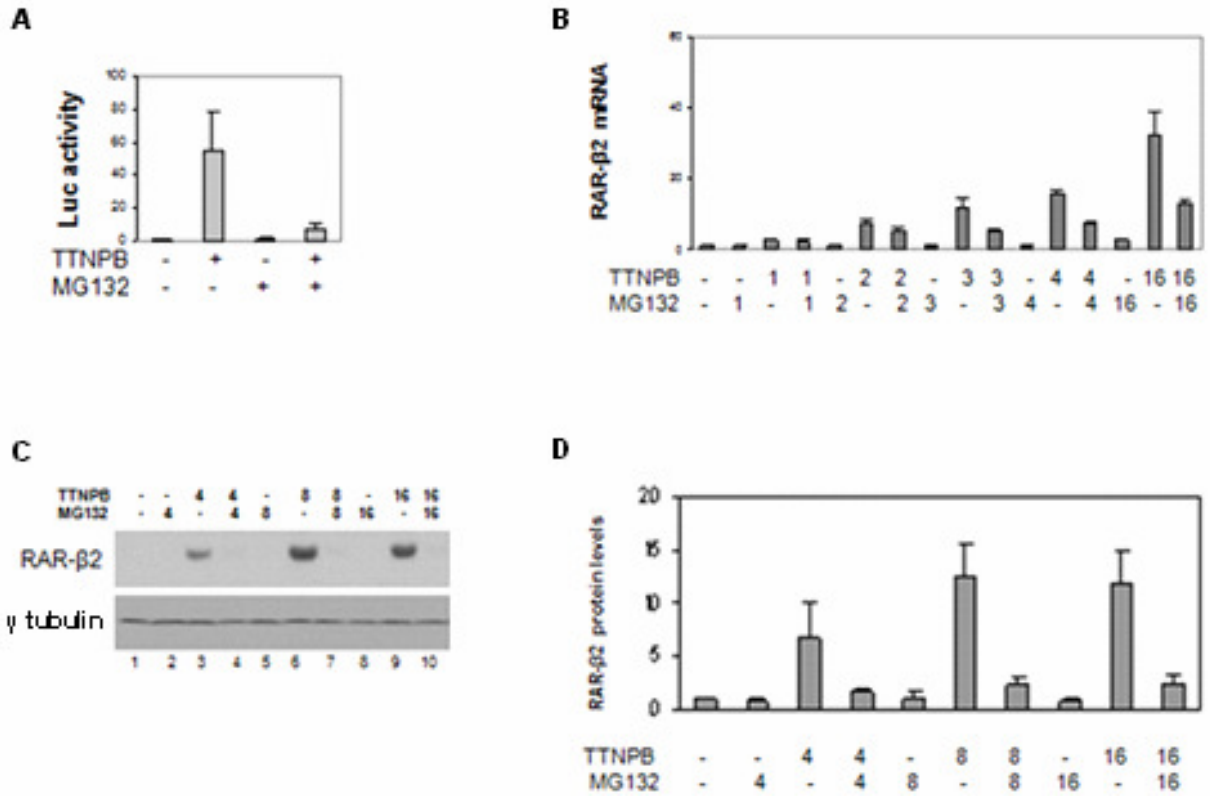

A
Luc activity
TTNPB
MG132
B
RAR-β2 mRNA
TTNPB - - 1 1 - 2 2 - 3 3 - 4 4 - 16 16
MG132 - 1 - 1 2 - 2 3 - 3 4 - 4 16 - 16
C
TTNPB - - 4 4 - 8 8 - 16 16
MG132 - 4 - 4 8 - 8 16 - 16
RAR-β2
γ tubulin
1 2 3 4 5 6 7 8 9 10
D
RAR-β2 protein levels
TTNPB - - 4 4 - 8 8 - 16 16
MG132 - 4 - 4 8 - 8 16 - 16

Figure 4.1 **The activity of the 26S proteasome is required for transactivating the RAR-β2 gene in P19 cells**

(A) Luciferase assay of P19 cells transfected with RAR-β2 reporters and respiratory syncytial virus β-Galactosidase (RSV-β-Gal) and treated with TTNPB (1 μM) ± MG132 (5 μM) for 16 hrs. Results are shown as fold inductions of the activities relative to untreated controls after being normalized to β-Galactosidase activity. Error bars represent the standard deviations of three independent experiments. (B) Quantitative real-time RT-PCR analysis of the RAR-β2 mRNA levels in P19 cells following 1, 2, 3, 4 and 16 hrs of TTNPB and/or MG132 treatments which are presented as fold variations compared to untreated control. Error bars represent the standard deviations of the triplicate from one experiment. (C) Western blot analysis of the RAR-β2 protein levels in P19 cells treated with TTNPB and/or MG132 for 4, 8 and 16 hrs in comparison to untreated control. The blots were stripped and reprobed with anti γ tubulin antibody (protein loading). (D) Quantitative analysis of panel C is expressed as fold variations compared to untreated cells after being normalized to the loading controls. Error bars represent the standard deviations of four independent experiments.

4.2 The activity of the 26S proteasome is crucial for the occupancy of RAR-β2 responsive promoter by ligand-activated RAR-α/RXR-α

In the present study, we investigated the role of the 26S proteasome in the occupancy of RAR-β2 promoters. The activity of the proteasome was shown to be involved in the recruitment of RNA Pol II to progesterone promoters (Dennis et al., 2005). However, its role in the occupancy of RAREs-containing promoters is not identified yet. We hypothesized here that the activity of the proteasome is required for the occupancy of RAR-β2-responsive promoters by liganded retinoid receptors/coactivators complexes. Therefore, the loss of this occupancy may participate in the mechanistic pathways by which proteasome inhibition represses RAR-β2 gene transactivation. Chromatin immunoprecipitation (ChIP) assays were performed to identify the consequences of proteasome inhibition on the occupancy of DR5 β2-RARE encompassing promoters. These assays were carried out in P19 cells treated with RAR-specific ligand TTNPB in the presence or absence of MG132 proteasome inhibitor for 1, 2 and 4 hours and in comparison to untreated control cells.

First, we studied the impact of proteasome inhibition on the occupancy of this promoter region by N-CoR. This protein is a RAR-β2 gene corepressor which must be removed from LBDs of retinoid receptors upon ligand binding to expose free surface for coactivators binding and hence RAR-β2 transactivation [reviewed in (Bastien and Rochette-Egly, 2004; Xu et al., 1999a)]. We revealed here that N-CoR was bound to RAR-β2-responsive promoters in control (lane 2) and in proteasome inhibitor MG132 treated only cells (lanes 3, 4 and 5) which increase with time of MG132 treatment. This binding was severely decreased or even absent in cells treated with RAR-specific ligand

TTNPB (lanes 6, 7 and 8) ± proteasome inhibitor MG132 (lanes 9, 10 and 11) (fig. 4.2 B & C). In conclusion, the occupancy of RAR-β2 promoters by N-CoR did not increase by proteasome inhibition of ligand-induced cells in comparison to ligand-induced cells only but increased with time in proteasome inhibition of unliganded cells. Thus, we cannot attribute the repressive effects of proteasome inhibition on RAR-β2 transactivation to an inability to release N-CoR from ligand-activated responsive promoters.

Next, ChIP assays were performed to demonstrate the occupancy of the RAR-β2 promoter regions by retinoid receptors coactivators including p300 and SRC-1. We found that there was ligand-dependent occupancy of RARE by SRC-1 as early as 1 hour (lane 6). This occupancy increases with extended time of RAR-specific ligand activation (lanes 6, 7 and 8). Moreover, SRC-1 occupancy of these promoters was decreased in a time dependent manner with addition of proteasome inhibitor to RAR-specific ligand (lanes 9, 10 and 11). This reduction had reached to complete loss within 4 hours (lane 11) (fig. 4.2 B). Moreover, RARE occupancy by p300 was evident at two hour following RAR ligand induction (lane 7) and increased further at 4 hours (lane 8). This occupancy was decreased with concomitant treatment for 2 hours (lane 10) and completely lost within 4 hours (lane 11). All together, these data reveal that proteasome inhibition for 4 hours severely represses or even abolishes the occupancy of ligand-induced P19 cells by retinoid receptors coactivators.

We were then interested in determining the basis underlying this loss of β2-responsive promoters occupancy by coactivators. We performed ChIP assays to investigate the effects of proteasome inhibition on the occupancy of β2-RARE by both RAR-α and RXR-α. We found that there was a significant decrease in the occupancy of

RARE by liganded RAR-α and RXR-α with proteasome inhibition in a time dependent manner. This decrease started within 2 hours (lane 10) of proteasome inhibition in RAR-specific ligand-induced cells (significant decrease with both RAR-α and RXR-α) and went to a complete loss within 4 hours (lane 11) (highly significant decrease with RAR-α and significant decrease with RXR-α) when compared to 1 hour of cotreatment (lane 9) (fig. 4.2 B & C). The occupancy of these promoters by retinoid receptors in cells treated only with proteasome inhibitor MG132 (lanes 3, 4 and 5) or with RAR-specific ligand induction only (lanes 6, 7 and 8) did not show any significant change in comparison to control cells (lane 2) (fig. 4.2 B & C). Moreover, the occupancy of RARE by RNA Pol II was not affected by the inhibition of the 26S proteasome activity either alone (lanes 3, 4 and 5) or in combination with ligand induction (lanes 9, 10 and 11) (fig. 4.2 D). Also, there was no demonstrated occupancy of the Exon 3 region by RNA Pol II within the time course of ChIP assays in our system (fig. 4.2 D).

Taken together, these data reveal that the activity of the proteasome is required for preserving the occupancy of RAREs by liganded retinoid receptors and their coactivators which is not due to preserving N-CoR occupancy to these regions. Furthermore, the recruitment of RNA Pol II to these responsive promoters is not proteasome-dependent.

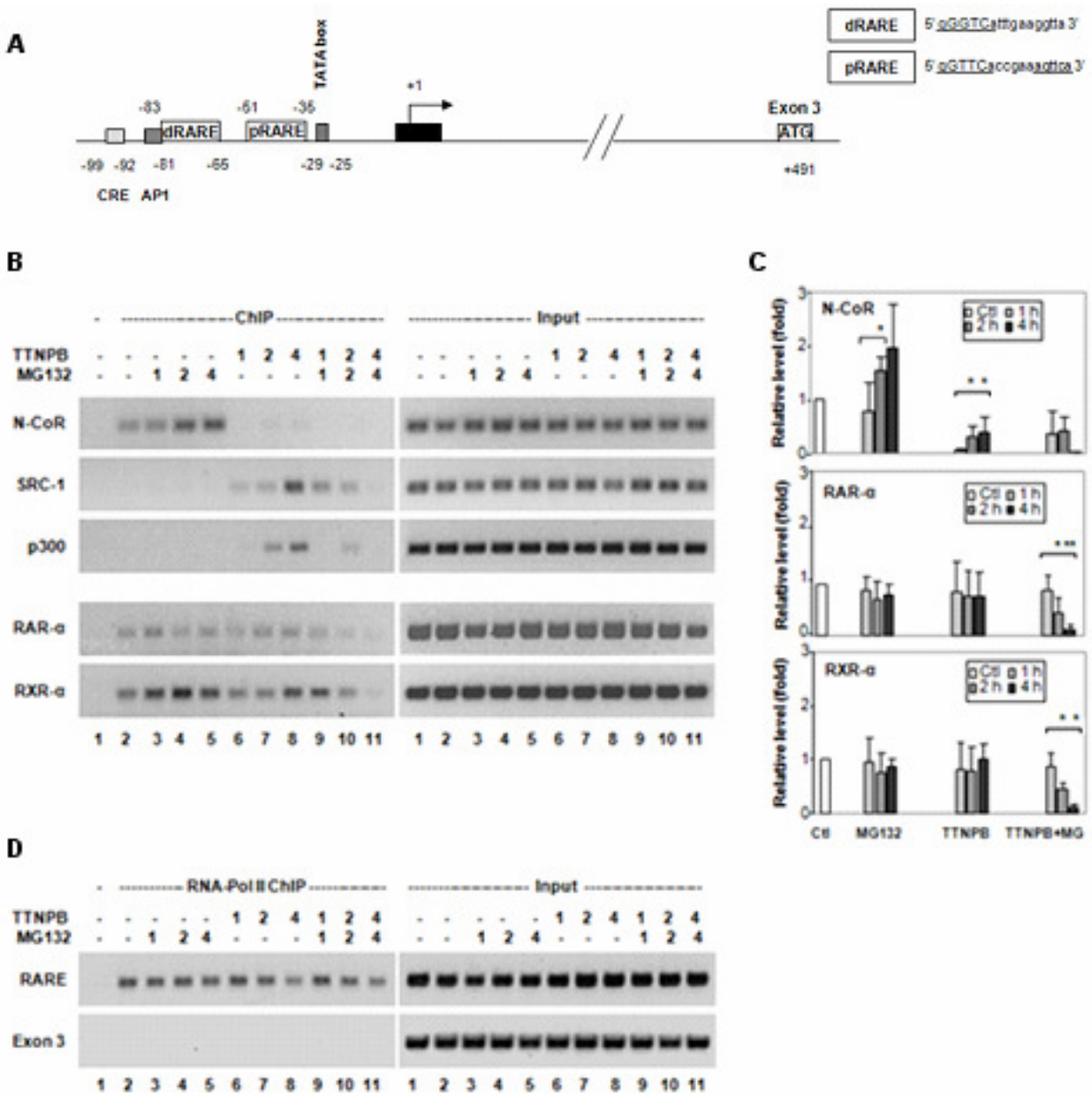

A
TATA box
+1
-83
-61
-35
dRARE
pRARE
Exon 3
ATG
-99 -92 -81 -65 -29 -25
+491
CRE AP1
dRARE 5' gGGTCattgaaggtta 3'
pRARE 5' gGTTCaccgaaagttca 3'
B
ChIP
Input
TTNPB
MG132
N-CoR
SRC-1
p300
RAR-α
RXR-α
1 2 3 4 5 6 7 8 9 10 11
C
N-CoR
RAR-α
RXR-α
Relative level (fold)
Ctl 1 h 2 h 4 h
Ctl MG132 TTNPB TTNPB+MG
D
RNA Pol II ChIP
Input
RARE
Exon 3

Figure 4.2 **The 26S proteasome function is important for maintaining the occupancy of β2-RAREs by liganded RAR-α/RXR-α/coactivators complexes**

(A) Schematic presentation of the RAR-β2 response elements at the promoter region. (B) ChIP analysis of the occupancy of RARE within RAR-β2 gene promoter by N-CoR, SRC-1, p300, RAR-α and RXR-α in P19 cells treated with TTNPB (1 μM) and/or MG132 (5 μM) for 1, 2 and 4 hrs in comparison to untreated control cells. The right panels represent the input control. (C) Quantitative analysis of N-CoR, RAR-α and RXR-α binding to RARE as described in panel (B) which are presented as fold variations in comparison to untreated controls and after being normalized to the inputs. Error bars represent standard deviations of three independent experiments (*$p \leq 0.05$ &**$p \leq 0.005$). (D) ChIP analysis to the occupancy of the RARE and the Exon 3 regions within the RAR-β2 gene promoter by RNA Pol II with the same treatment conditions as in panel (B). Input control is in the right panels. Lane 1 in all panels represents control cells with no antibody. The ChIP assays were repeated at least three independent times.

4.3 The loss of ligand-dependent β2-RAREs occupancy is determined by the duration of proteasome inhibition

Here, we aimed to identify if the duration of RAR-specific ligand activation is responsible for the time point at which proteasome inhibition consequences on promoter occupancy appear or it is the duration of proteasome inhibition *per se*. Therefore, we designed a protocol for pretreating P19 cells with MG132 proteasome inhibitor for either 2 or 3 hours before adding the RAR-specific ligand TTNPB to these proteasome inhibited cells for an additional 1 hour (fig. 4.3 A). ChIP analysis was then performed using antibodies against RAR-α and RXR-α in P19 cells treated according to this protocol. We found that there was loss in the occupancy of RARE as early as 1 hour following TTNPB treatment with 2 and 3 hours of MG132 pretreatment (lanes 7 and 8) in comparison to untreated cells (lane 2), cells treated with MG132 only for 3 or 4 hours (lanes 3 and 4) and cells treated with TTNPB ± MG132 for 1 hour (lanes 5 and 6) (fig. 4.3 B). Accordingly, we can conclude that at least 3 hours of proteasomal inhibition is required to disrupt the occupancy of RARE by ligand-induced retinoid receptors. This means that the duration of proteasome inhibition *per se* is the determinant for the perturbed ligand-dependent occupancy of β2-RARE by retinoid receptors.

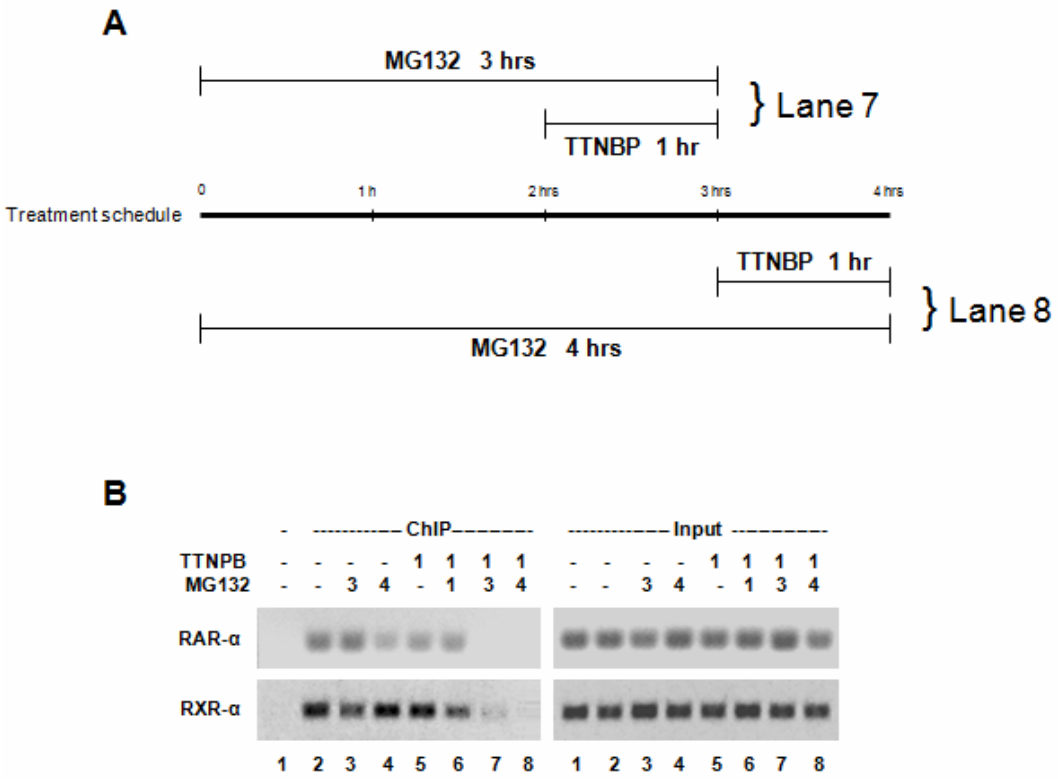


Figure 4.3 **The loss of β2-RAREs occupancy by ligand-induced retinoid receptors is dependent on the duration of proteasome inhibition *per se***

(A) Schematic illustration of the pretreatment schedule with MG132. (B) ChIP analysis of the occupancy of RARE with RAR-α and RXR-α in P19 cells pretreated with MG132 (5 μM) for 2 and 3 hrs as illustrated then induced with TTNPB (1 μM) for additional 1 hr (lanes 7 and 8), cells treated only with TTNPB for 1 hr (lane 5) or MG132 for 3 and 4 hrs (lanes 3 and 4) or cotreated with TTNPB and MG132 for 1 hr (lane 6) in comparison to untreated control cells (lane 2). Lane 1 represents untreated control cells without antibody. The right panels represent the input control. The ChIP assays were repeated at least three independent times.

4.4 The role of the proteasome in ligand-dependent occupancy of RARE is restricted to responsive promoters in the vicinity of TATA box

First, we examined whether the function of the 26S proteasome in transactivating RAR-regulated genes in P19 cells is restricted to the RAR-β2 gene or other RA-responsive genes are affected as well. Thus, we investigated the consequences of proteasome inhibition using MG132 on the expression of another RAR-regulated gene, Cyp26A1. Quantitative real time RT-PCR analysis was performed to measure Cyp26A1 mRNA levels in P19 cells treated with the RAR-specific ligand TTNPB and/or with MG132 for 1 and 4 hours in comparison to untreated cells. We found that there was a large induction of Cyp26A1 mRNA transcripts within 4 hours of treatment with TTNPB which was reduced to approximately one third when cells were treated with MG132 and TTNPB (fig. 4.4 A). Thus, the function of the proteasome is vital for transactivating the Cyp26A1 gene.

Next, we used two RAREs within the Cyp26A1 gene to study the effects of proteasome inhibition on their ligand-dependent occupancy. These response elements are the DR5 proximal R1 and distal R2 regions. ChIP assays were performed using antibodies against RAR-α and RXR-α in P19 cells treated as described above in real time RT-PCR. We found that the occupancy of the R1 regions by liganded RAR-α and RXR-α was lost within 4 hours of concomitant treatment (lane 8) in comparison to untreated control cells (lane 2) or cells treated only with either TTNPB (lanes 5 and 6) or MG132 (lanes 3 and 4) (fig. 4.4 C & D). These findings were similar to what were shown with RAR-β2 response elements. In contrast, the occupancy of the R2 regions with liganded

RAR-α or RXR-α was not disrupted by proteasomal inhibition for 4 hours (lane 8) as the R1 region (fig. 4.4 C & D).

Collectively, these data indicate that the function of the proteasome is required for preserving the ligand-dependent occupancy of RAREs which are only in close proximity to the TATA box. Therefore, the context of the retinoid-responsive DNA regulatory regions determines the function of the proteasome in ligand-dependent occupancy of RARE.

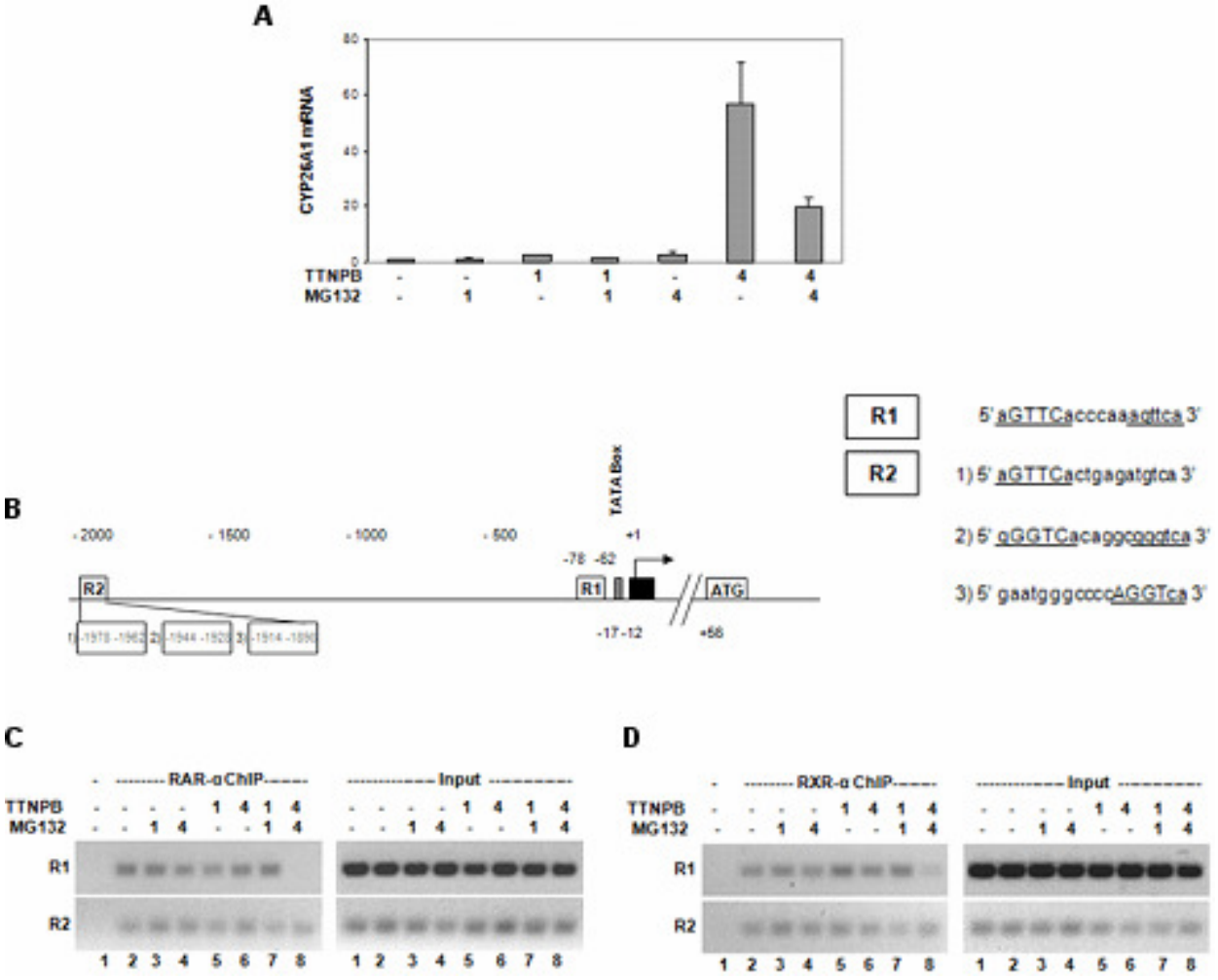

A
CYP26A1 mRNA
TTNPB
MG132
B
TATA Box
R2
R1
ATG
R1
R2
5' aGTTCacccaaagttca 3'
1) 5' aGTTCactgagatgtca 3'
2) 5' gGGTCacaggcgggtca 3'
3) 5' gaatgggcccgAGGTca 3'
C
RAR-α ChIP
Input
TTNPB
MG132
R1
R2
D
RXR-α ChIP
Input
TTNPB
MG132
R1
R2

Figure 4.4 **The proteasome activity is needed for preserving the occupancy of R1 RARE within the Cyp26A1 gene by liganded RAR-α/RXR-α but not R2 RARE**

(A) Quantitative Real time RT-PCR analysis of Cyp26A1 mRNA levels in P19 cells treated for 1 and 4 hrs with MG132 (5 μM) and/or TTNPB (1 μM) in comparison to untreated cells. Data are presented as fold variations compared to untreated control. Error bars represent the standard deviations of the triplicate from one experiment. (B) Schematic presentation of the Cyp26A1 R1 and R2 regions. (C & D) ChIP analysis to the occupancy of the Cyp26A1 R1 and R2 regulatory regions by RAR-α and RXR-α respectively in P19 cells treated for 1 and 4 hrs with TTNPB (1 μM) and/or MG132 (5 μM) in comparison to untreated controls. The right panels represent the input control. The first lane in all ChIP panels represents no antibody controls. The ChIP assays were repeated at least three independent times.

4.5 Long term proteasome inhibition does not affect the ligand-dependent occupancy of the R2 region within the Cyp26A1 gene

To distinguish if the occupancy of the R2 region within the Cyp26A1 gene by liganded RAR-α will be lost later on with prolonged duration of proteasome inhibition, we performed ChIP analysis with extended time of proteasome inhibition. P19 cells were treated for 8 hours with RAR-selective ligand TTNPB in the presence or absence of MG132 proteasome inhibitor and in comparison to untreated control cells. Next, ChIP assays were conducted using antibodies against RAR-α. Once more, we found that the occupancy of the R2 region by RAR-α was not disrupted with 8 hours of concomitant treatments which was not the case regarding the R1 region occupancy (lane 8) (fig. 4.5). Therefore, we can conclude that the occupancy of R2 but not R1 RAREs by liganded RAR-α is not affected by proteasome inhibition even with expanded duration of this inhibition.

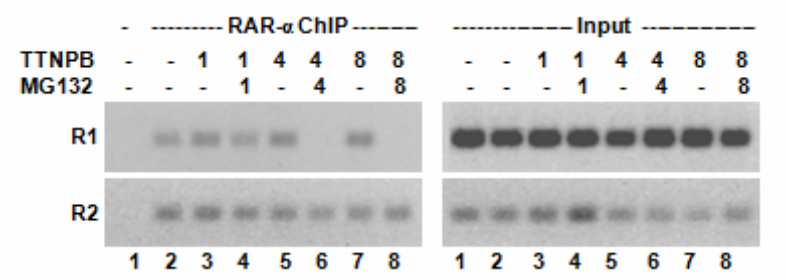


Figure 4.5 **The ligand-dependent occupancy of the R2 region is proteasome-independent even with extended proteasome inhibition**

ChIP analysis to the occupancy of the Cyp26A1 R1 and R2 regions by RAR-α in P19 cells treated with TTNPB (1 μM) ± MG132 (5 μM) for 1, 4 and 8 hrs in comparison to untreated cells. The right panels represent the input control. The first lane represents no antibody controls. These ChIP assays were repeated three independent times.

4.6 The consequences of proteasome inhibition on RAR-regulated genes expression are not specific to a single proteasome inhibitor

To investigate if the effects of proteasomal inhibition on the expression of RA-responsive genes are limited to MG132 proteasome inhibitor or not, we used bortezomib (PS-341) proteasome inhibitor from the peptide boronates group of proteasome inhibitors. This proteasome inhibitor is a more specific, more stable and more potent proteasome inhibitor than MG132 (Adams, 2003). First, we performed Western blot assays to measure RAR-β2, RAR-α and RXR-α protein levels in P19 cells treated with the RAR-selective ligand and/or with PS-341 to detect the least effective concentration of this inhibitor. We found that PS-341 (10 nM) (lanes 5 and 10) is the lowest effective dose that had a repressive effects on RAR-β protein levels but without altering RAR-α or RXR-α protein levels. Thus, this concentration was used in further experiments (fig. 4.6 A). After that, P19 cells were treated for 1 and 4 hours with PS-341 proteasome inhibitor and/or RAR-selective ligand TTNPB in comparison to control cells. Next, quantitative real time RT-PCR (fig. 4.6 B) and ChIP assays (fig. 4.6 C & D) were performed in these cells to measure both RAR-β2 and Cyp26A1 mRNA levels as well as to study the occupancy of their response elements by RAR-α/RXR-α respectively. We found that PS-341 has the same effects as those demonstrated with MG132 proteasome treatment on both genes and with both quantitative RT-PCR and ChIP assays. Taken together, our data reveal that the effects of proteasome inhibition on RAR-β2 and Cyp26A1 genes expression as well as their promoters occupancy is not restricted to a specific type of proteasome inhibitors.

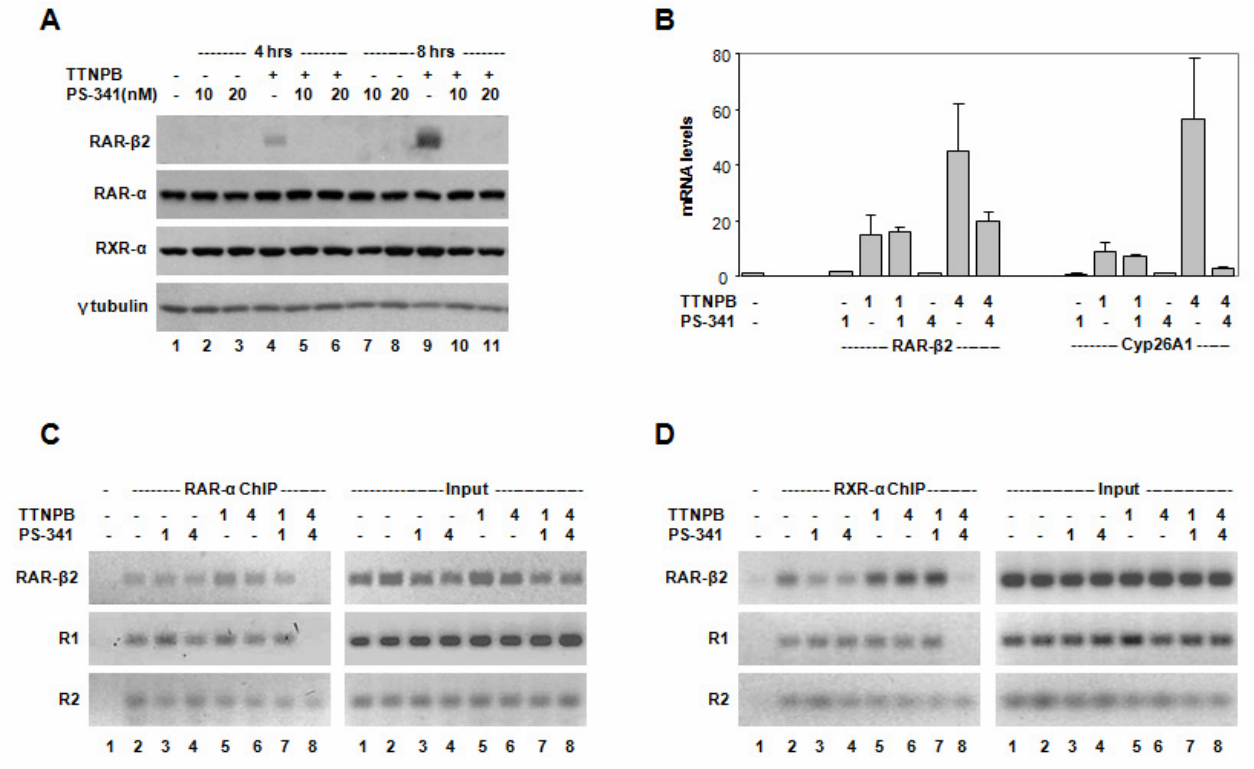

A
4 hrs
8 hrs
TTNPB
PS-341(nM)
RAR-β2
RAR-α
RXR-α
γtubulin
1 2 3 4 5 6 7 8 9 10 11
B
mRNA levels
80
60
40
20
0
TTNPB
PS-341
RAR-β2
Cyp26A1
C
RAR-α ChIP
Input
TTNPB
PS-341
RAR-β2
R1
R2
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
D
RXR-α ChIP
Input
TTNPB
PS-341
RAR-β2
R1
R2
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8

Figure 4.6 **The consequences of PS-341 proteasome inhibitor on the RAR-β2 and Cyp26A1 genes expression and on their RAREs occupancy**

(A) Western blot analysis of the RAR-β2 protein levels in P19 cells treated with 10 or 20 nM of PS-341 and/or TTNPB (1 μM) for 4 and 8 hrs in comparison to untreated control cells. The blots were stripped and reprobed with anti RAR-α, anti RXR-α and anti γ tubulin antibodies. The Western blots were repeated three independent times. (B) Quantitative real-time RT-PCR analysis to measure both RAR-β2 and Cyp26A1 mRNA levels in P19 cells treated for 1 and 4 hrs with PS-341 (10 nM) and/or TTNPB (1 μM) in comparison to untreated cells. Results are presented as fold variations in comparison to untreated control cells. Error bars represent the standard deviations of the triplicate from one experiment. (C & D) ChIP analysis to the occupancy of β2, R1 and R2 RAREs by RAR-α and RXR-α in P19 cells treated with TTNPB (1 μM) and/or PS-341 (10 nM) for 1 and 4 hrs in comparison to untreated control. The right panels are input controls. Lane 1 in all ChIP panels represents controls with no antibody. The ChIP assays were repeated three independent times.

4.7 The occupancy of Cyp26A1 response elements by either RNA Pol II or TFIIH is proteasome-independent

Next, we were interested in identifying the molecular mechanisms underlying the difference between the effects of proteasome on the ligand-dependent occupancy of the R1 and the R2 regions within the Cyp26A1 gene. Both RNA Pol II and TFIIH are recruited to the TATA box. It was reported that RNA Pol II was recruited to both R1 and R2 regions of the Cyp26A1 gene (Bruck et al., 2009). Additionally, it was shown that there is a Cyp26A1 gene looping to make a bridge that connects the R1 with the R2 regions within this gene (Bruck et al., 2009). Moreover, the occupancy of the R1 region by TFIIH was reported before in resting (non ligand activated) F9 cells and increases with ligand activation (Bruck et al., 2009). However, the recruitment of TFIIH to the R2 region was reported in the same study only with ligand activation and to a lesser extent than its recruitment to the R1 region (Bruck et al., 2009). Therefore, we aimed to examine the occupancy of the R1 and R2 regions by both RNA Pol II and TFIIH through ChIP assays. P19 cells were treated with RAR-specific ligand TTNPB and/or MG132 proteasome inhibitor for 1 and 4 hours in comparison to untreated cells. ChIP assays were then performed using antibodies against RNA Pol II. Similar to what was shown before in the Bruck et al. study in 2009 (Bruck et al., 2009), we demonstrated that both R1 and R2 regions were occupied by RNA Pol II in the control (lane 2) and TTNPB treated cells (lanes 5 and 6). As well, the inhibition of the proteasome function did not affect the pattern of RNA Pol II occupancy to these regions (lanes 3, 4, 7 and 8) (fig. 4.7 A). Moreover, P19 cells were treated with RAR-specific ligand TTNPB in the presence or absence of either MG132 or PS-341 proteasome inhibitors for 1, 2 and 4 hours and in

comparison to untreated control cells or cell treated only with either MG132 or PS-341. ChIP assays were then carried out using antibodies against TFIIH. We found that the inhibition of the proteasomal function did not affect the pattern of TFIIH occupancy to these regions which was not lost in both R1 and R2 regulatory regions (lanes 3-8 and 12-17). Also there was no significant change in the occupancy of TFIIH to these regions with ligand induction (lanes 9, 10 and 11) in comparison to control cells (lane 2) (fig. 4.7 B). Taken together, these data suggest that the proteasomal function has no influence on the occupancy of both R1 and R2 regions within Cyp26A1 gene with RNA Pol II as well as TFIIH. Therefore, these proteins may not play a direct role in mediating the Cyp26A1 gene transactivation functions of the proteasome and in the difference between R1 and R2 regions regarding their proteasome-dependent occupancy by liganded retinoid receptors

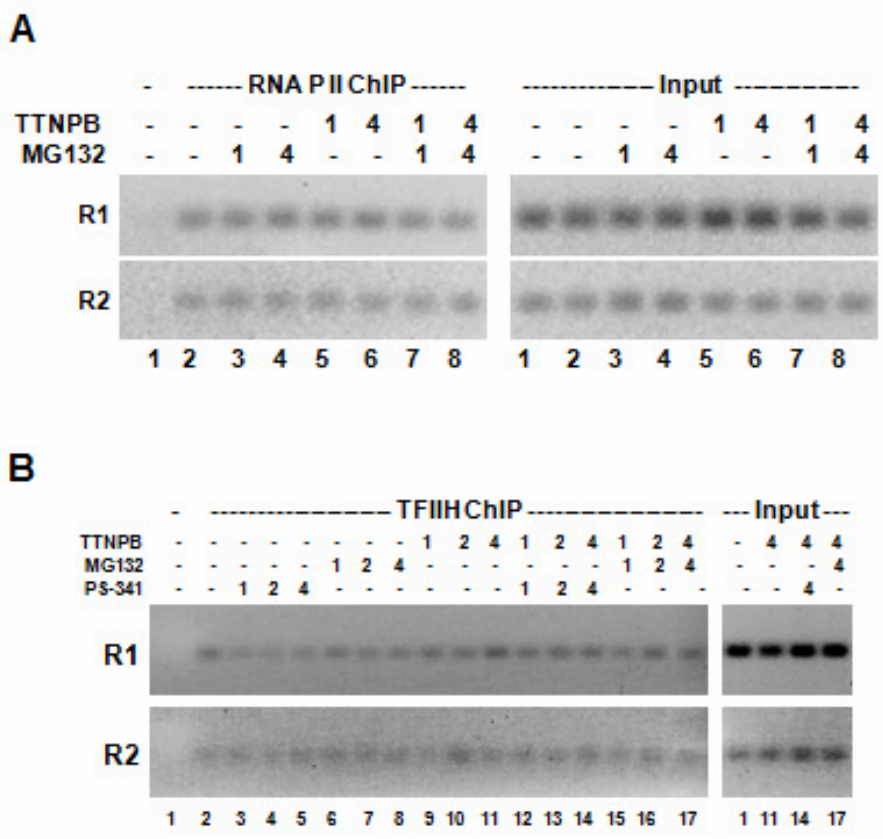

A
- ------ RNA P II ChIP ------ ---------- Input ----------
TTNPB - - - - 1 4 1 4 - - - - 1 4 1 4
MG132 - - 1 4 - - 1 4 - - 1 4 - - 1 4
R1
R2
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
B
- ---------- TFIIH ChIP ---------- --- Input ---
TTNPB - - - - - - - - 1 2 4 1 2 4 1 2 4 - 4 4 4
MG132 - - - - - 1 2 4 - - - - - - 1 2 4 - - - 4
PS-341 - - 1 2 4 - - - - - - 1 2 4 - - - - - 4 -
R1
R2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 1 11 14 17

Figure 4.7 **The occupancy of the R1 and the R2 regions within the Cyp26A1 gene by either RNA Pol II or TFIIH is not dependent on the proteasome activity**

(A) ChIP analysis to the occupancy of the Cyp26A1 R1 and R2 regions by RNA Pol II in P19 cells treated for 1 and 4 hrs with MG132 (5 μM) and/or TTNPB (1 μM) in comparison to untreated cells. The right panels represent the input control. First lane represents no antibody controls. (B) ChIP analysis to the occupancy of R1 and R2 RARE by TFIIH in P19 cells treated with TTNPB (1 μM) ± either MG132 (5 μM) or PS-341 (10 nM) for 1, 2 and 4 hrs in comparison to untreated control cells or cells treated with proteasome inhibitors only. The right panels are input controls. Lane 1 represents controls with no antibody. The ChIP assays were repeated three independent times.

4.8 The 26S proteasome activity is necessary for the association of liganded RAR-α with its coactivators

To address the effects of inhibition of the 26S proteasome on the association between RAR-α and both SRC-1 and p300, coimmunoprecipitation assays were performed. P19 cells were treated with RAR-specific ligand TTNPB and/or proteasome inhibitor MG132 for 4 hours. Cellular extracts were used for coimmunoprecipitation of RAR-α with these coactivators using anti SRC-1 and anti p300 antibodies for immunoprecipitation. Western blot analysis was done using antibodies against RAR-α then the membranes were reprobed with anti SRC-1 and anti p300 antibodies as controls. We found that both SRC-1 and p300 proteins were associated with RAR-α in cells treated only with TTNPB (lane 4) in comparison to control untreated cells (lane 2) or cells treated with either MG132 (lane 3) or concomitantly with MG132 plus TTNPB (lane 5) (fig. 4.8 A & B). These data reveal that the activity of the 26S proteasome is important for the association between DNA-free RAR-α and its coactivators.

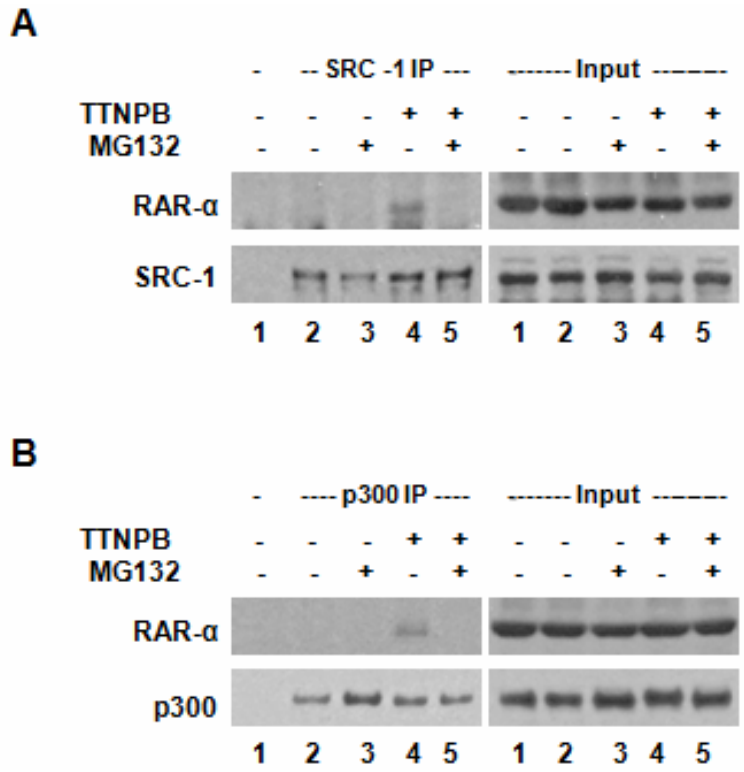


Figure 4.8 **The 26S proteasome activity is necessary for the association of ligand-induced RAR-α with SRC-1 and p300**

(A) Coimmunoprecipitation of endogenous RAR-α with SRC-1 in P19 cells treated for 4 hrs with TTNPB (1 μM) and/or MG132 (5 μM) in comparison to untreated cells. The blots were probed with anti RAR-α antibody then stripped and reprobed with anti SRC-1 antibody (IP protein). (B) Coimmunoprecipitation of endogenous RAR-α with p300 (IP protein) in cells treated for 4 hrs as in panel A. The blots were probed with anti RAR-α antibody then stripped and reprobed with anti p300 antibody. The coimmunoprecipitation experiments were repeated at least three times. The input represents 10% of the whole cell extracts used. Lane 1 in all panels represents control cells without antibody.

4.9 The ligand-dependent interaction of DNA-free RAR-α with RXR-α and of RXR-α with its coactivators in solution is proteasome-dependent

To delineate the implications of proteasome inhibition on the interaction between RAR-α and RXR-α in solution, P19 cells were treated with RAR-selective ligand TTNPB and/or MG132 proteasome inhibitor for 4 hours. Cellular extracts were used for coimmunoprecipitation of RAR-α with RXR-α using anti RXR-α antibody for IP. Western blot analysis was done using antibodies against RAR-α. Then the membranes were reprobed with anti RXR-α antibody as control. Analysis of the immunoprecipitants showed that cells treated with TTNPB only (lane 4) showed a clear association of RAR-α with RXR-α which was significantly decreased or even lost when MG132 was added to TTNPB treatment (lane 5) (fig. 4.9 A). Additionally, P19 cells were treated as in fig. 4.8 but cellular extracts this time were used for coimmunoprecipitation of SRC-1, p300 or N-CoR with RXR-α using anti SRC-1, anti p300 or anti N-CoR antibodies for IPs. We found that both SRC-1 and p300 proteins were associated with RXR-α in cells treated only with TTNPB (lane 4) in comparison to control untreated cells (lane 2) and cells treated with either MG132 only (lane 3) or concomitantly with MG132 plus TTNPB (lane 5) (fig. 4.9 B). Also, we found that N-CoR was associated with RXR-α in the absence of ligand (lanes 2 and 3) but not with ligand induction either alone (lane 4) or with MG132 (lane 5) treatment (fig. 4.9 C). All together, these data suggest that the DNA free ligand-dependent association between RAR-α/RXR-α and hence between both receptors and their coactivators are proteasome-dependent. Also, the pattern of RXR-α binding to N-CoR did not change with proteasome inhibition i.e. there was no interaction of corepressors with RXR-α upon ligand induction.

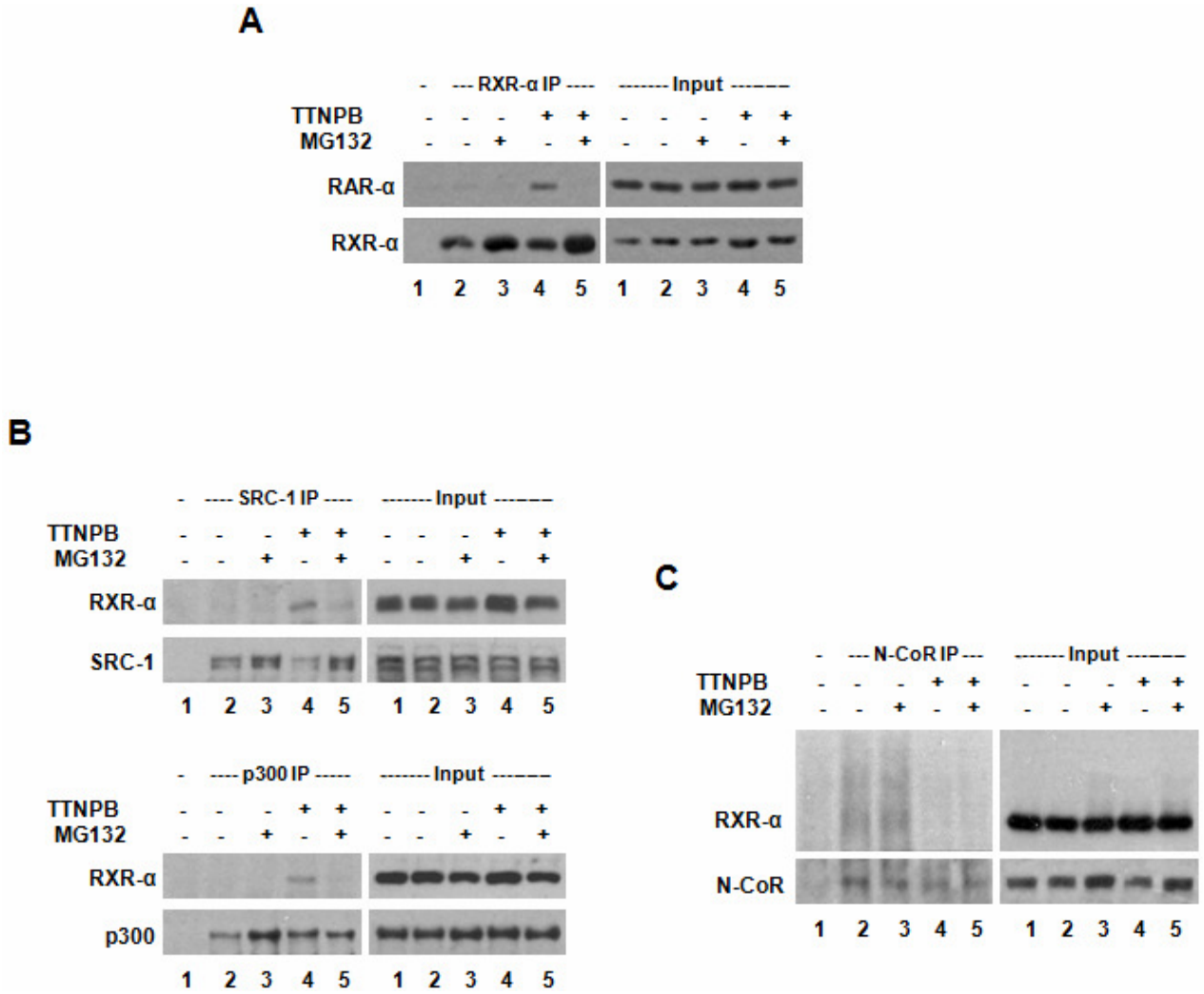

A
- --- RXR-α IP ---- ------- Input -------
TTNPB - - - + + - - - + +
MG132 - - + - + - - + - +
RAR-α
RXR-α
1 2 3 4 5 1 2 3 4 5
B
- ---- SRC-1 IP ---- ------- Input -------
TTNPB - - - + + - - - + +
MG132 - - + - + - - + - +
RXR-α
SRC-1
1 2 3 4 5 1 2 3 4 5
- ---- p300 IP ----- ------- Input -------
TTNPB - - - + + - - - + +
MG132 - - + - + - - + - +
RXR-α
p300
1 2 3 4 5 1 2 3 4 5
C
- --- N-CoR IP --- ------- Input -------
TTNPB - - - + + - - - + +
MG132 - - + - + - - + - +
RXR-α
N-CoR
1 2 3 4 5 1 2 3 4 5

Figure 4.9 **The proteasome activity is necessary for association of liganded RAR-α with RXR-α and of RXR-α with its coactivators in solution upon ligand activation**

(A) Coimmunoprecipitation of the endogenous RXR-α with RAR-α in P19 cells treated with TTNPB (1 μM) and/or MG132 (5 μM) for 4 hrs in comparison to untreated control cells. Western blots were first probed with anti RAR-α antibody then stripped and reprobed with anti RXR-α antibody (IP protein). (B) Coimmunoprecipitation of endogenous RXR-α with SRC-1 and p300 in cells treated for 4 hrs as in panel A. The blots were probed with anti RXR-α antibody then stripped and reprobed with anti SRC-1 (IP) and anti p300 (IP) antibodies. (C) The same as in panel B except that the IP protein was N-CoR and Western blots were first probed with RXR-α antibody then reprobed with anti N-CoR antibody. All coimmunoprecipitation experiments were repeated at least three times. The input represents 10% of the whole cell extracts used. Lane 1 in all panels represents control cells but without IP.

4.10 The subcellular localization of RAR-α in P19 cells is both ligand- and proteasome-dependent

It had been reported that the subcellular localization of retinoid receptors is not restricted to the nucleus as what was believed before but may be cytoplasmic as well (Huang et al., 2008; Tahayato et al., 1993; Weis et al., 1994). Moreover, the subcellular distribution of retinoid receptors was shown to be different from one cell line to another and from one retinoid receptors subtype to another (Dufour and Kim, 1999). There are many factors including ligand induction and protein kinase C that have been shown to regulate the subcellular trafficking of different retinoid receptors subtypes in certain cell lines (Huang et al., 2008; Tahayato et al., 1993; Weis et al., 1994). However, the role of the 26S proteasome in regulating retinoid receptors subcellular localization remains unclear.

To detect the effects of proteasome inhibition on the subcellular localization of retinoid receptors, immunofluorescent staining for RAR-α was performed in P19 cells treated for 4 hours with RAR-specific ligand TTNPB and/or proteasome inhibitor MG132 in comparison to untreated cells. We used hoechst 33258 pentahydrate to stain DNA. We found that proteasome inhibition induces the localization of RAR-α within perinuclear cytoplasmic foci in comparison to control cells. Indeed, the number of these foci per cell was higher (highly significant) with proteasome inhibition of liganded RAR-α (average 2.5) than the number of foci per cell with proteasomal inhibition of non activated receptors (average 1.5) (fig. 4.10 A & B). These foci appear within 2 hours of proteasome inhibition either alone or with RAR ligand induction (data not shown). RAR-α was observed within nuclear microfoci in RAR ligand-activated cells when compared

to untreated control cells (fig. 4.10 A). These microfoci were observed before with a number of nuclear receptors upon ligand induction in different cell lines e.g. ERs, ARs, GRs and RXR-α (Tanaka et al., 2004). Immunofluorescent ApoTome microscopy was used to visualize these cells. These data suggest that proteasome activity is required for maintaining the nuclear localization of RAR-α and hence the subcellular localization of RAR-α in P19 cells is proteasome-dependent.

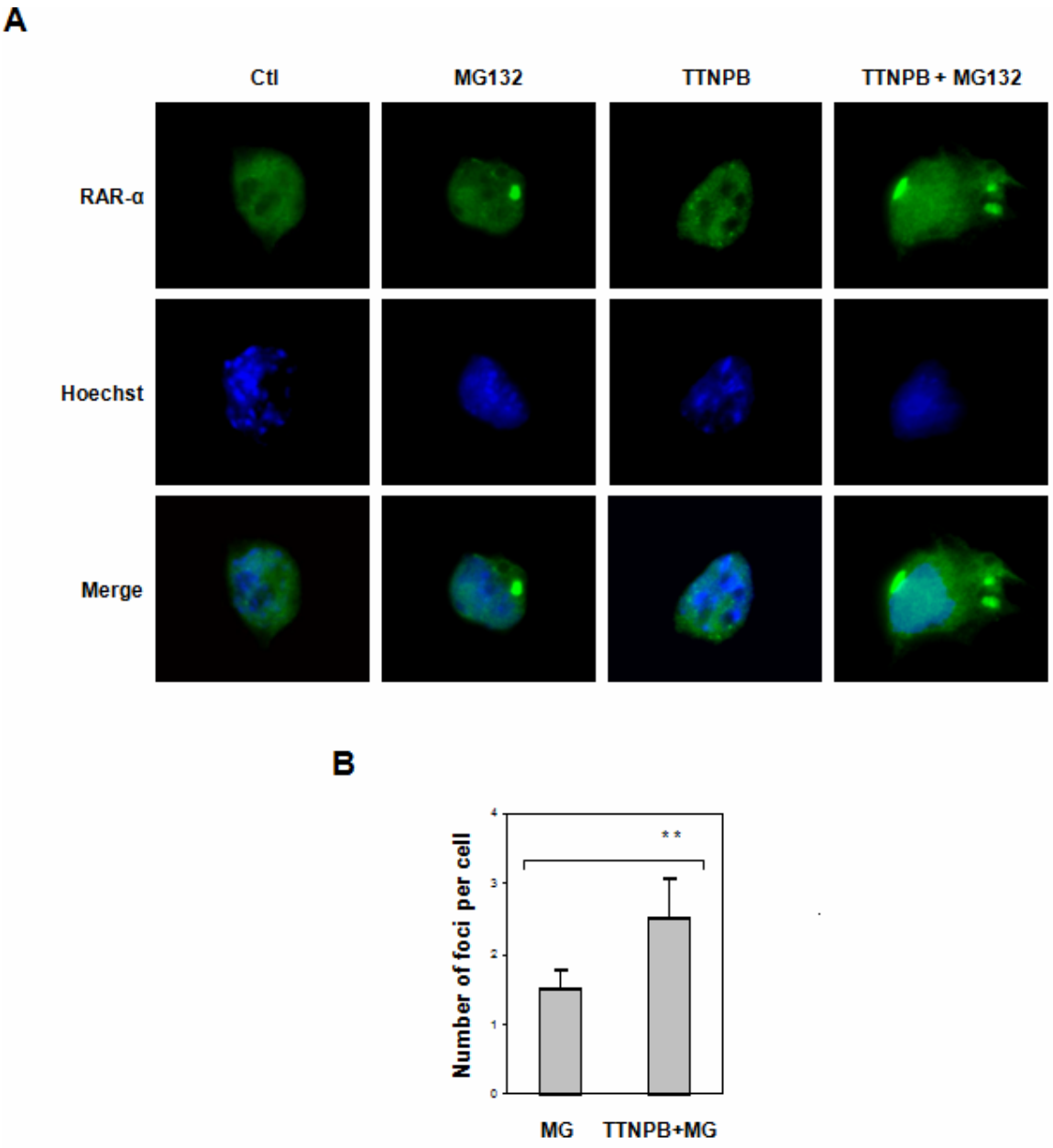

A
Ctl
MG132
TTNPB
TTNPB + MG132
RAR-α
Hoechst
Merge
B
Number of foci per cell
**
MG
TTNPB+MG

Figure 4.10 **Inhibition of the 26S proteasome activity induces the accumulation of RAR-α within cytoplasmic foci in P19 cells**

(A) Immunofluorescent microscopy with ApoTome optical sectioning was used to visualize P19 cells treated with TTNPB (1 μM) and/or MG132 (5 μM) for 4 hrs then stained with RAR-α antibody (green) and hoechst (blue) followed by secondary staining with goat anti-rabbit Alexa Fluor® 488 in comparison to untreated control cells. (B) Quantitative analysis of the number of foci per cell cotreated with TTNPB plus MG132 in comparison to cells treated with MG132 only. Error bars represent the standard deviation of at least eight independent experiments (**$p \leq 0.005$).

4.11 The subcellular localizations of RXR-α, p300 and SRC-1 are restricted to the nucleus in P19 cells

Next, we aimed to identify if these proteasome-dependent cytoplasmic foci are due to inadequate protein fixation as mentioned before in (Yamashita, 1998) report or not. To conduct this aim, we performed immunofluorescent staining for RXR-α, p300 and SRC-1 in P19 cells fixed by the same method and treated as in fig. 4.10 (see materials and methods for fixation method) then immunofluorescent staining was carried out in these cells using anti RXR-α, anti SRC-1 or anti p300 antibodies. We found that there is no change in the subcellular localization (they are still nuclear) of RXR-α (data not shown), SRC-1 (data not shown) and p300 (fig. 4.11) with RAR-specific ligand induction, proteasome inhibition or concomitant treatment with both of them in comparison to control cells. Therefore, the cytoplasmic localization of RAR-α in our study is not a result of fixation method defects. Taken together, our data suggest that the activity of the 26S proteasome has no influence on the localization of RXR-α and its coactivators in P19 cells which are restricted to the nucleus. Thus, a possibility that a change in the subcellular localization of retinoid receptors coactivators or of RXR-α with proteasome inhibition could be one of the mechanisms that underlie the function of proteasome in transactivating RA-responsive genes is excluded.

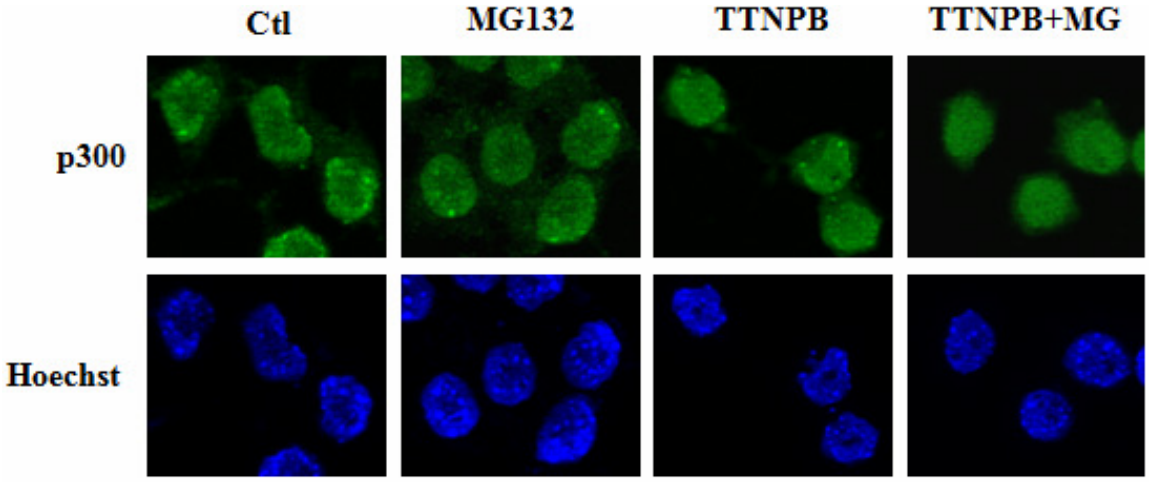


Figure 4.11 **Inhibition of the 26S proteasome activity does not affect the subcellular localization of p300 in P19 cells**

Immunofluorescence staining was used to determine any changes in p300 proteins localization in relation to the nucleus in P19 cells treated with MG132 (5 μM) and/or TTNPB (1 μM) for 4 hrs in comparison to untreated control cells. Anti-p300 antibody was used with green fluorescence labeled secondary antibody (green). Hoechst staining was used to visualize the nucleus (blue). ApoTome microscopy was used to take several sections within the nucleus. Shown are representatives of single tomography. The experiment was repeated at least three times.

4.12 A RAR-selective Ligand enhances the ubiquitination of RAR-α in P19 cells

It was established that ligand activation induces retinoid receptors ubiquitination (Boudjelal et al., 2000; DeMartino and Slaughter, 1999; Gianni et al., 2002; Gianní et al., 2003; Kopf et al., 2000; Tanaka et al., 2001b; Zhu et al., 1999a). However, whether or not this ubiquitination is only for degrading retinoid receptors, or for homeostasis of these receptors as well is not known. Therefore, additional investigations need to be performed to identify the exact role of this ubiquitination in regulating retinoid receptor-activated genes.

To demonstrate the effects of RAR ligand activation on the ubiquitination of retinoid receptors, we performed an *in vivo* ubiquitination assay. P19 cells were first treated for 4 hours with proteasome inhibitor MG132 alone or in addition to RAR-selective ligand TTNPB then specific antibodies against RAR-α and RXR-α were used to immunoprecipitate these receptors. Western blot assays were performed to measure the ubiquitin proteins conjugated with the immunoprecipitated RAR-α and RXR-α proteins. The membranes were first probed with anti-ubiquitin antibodies then stripped and reprobed with anti-RAR-α and anti-RXR-α antibodies as internal controls. We found that there is significant three fold greater enhancement of ubiquitin/RAR-α conjugation in cotreated cells (lane 5) when compared with MG132 treated only cells (lane 3) (fig. 4.12 A & B). The enhancement of ubiquitin/RXR-α conjugation in cotreated cells (lane 5) was not significant and less than 2 fold in comparison to MG132-treated cells (lane 3) (fig. 4.12 C & D). In conclusion, our data suggest that the ubiquitination of RAR is enhanced in a ligand-dependent manner.

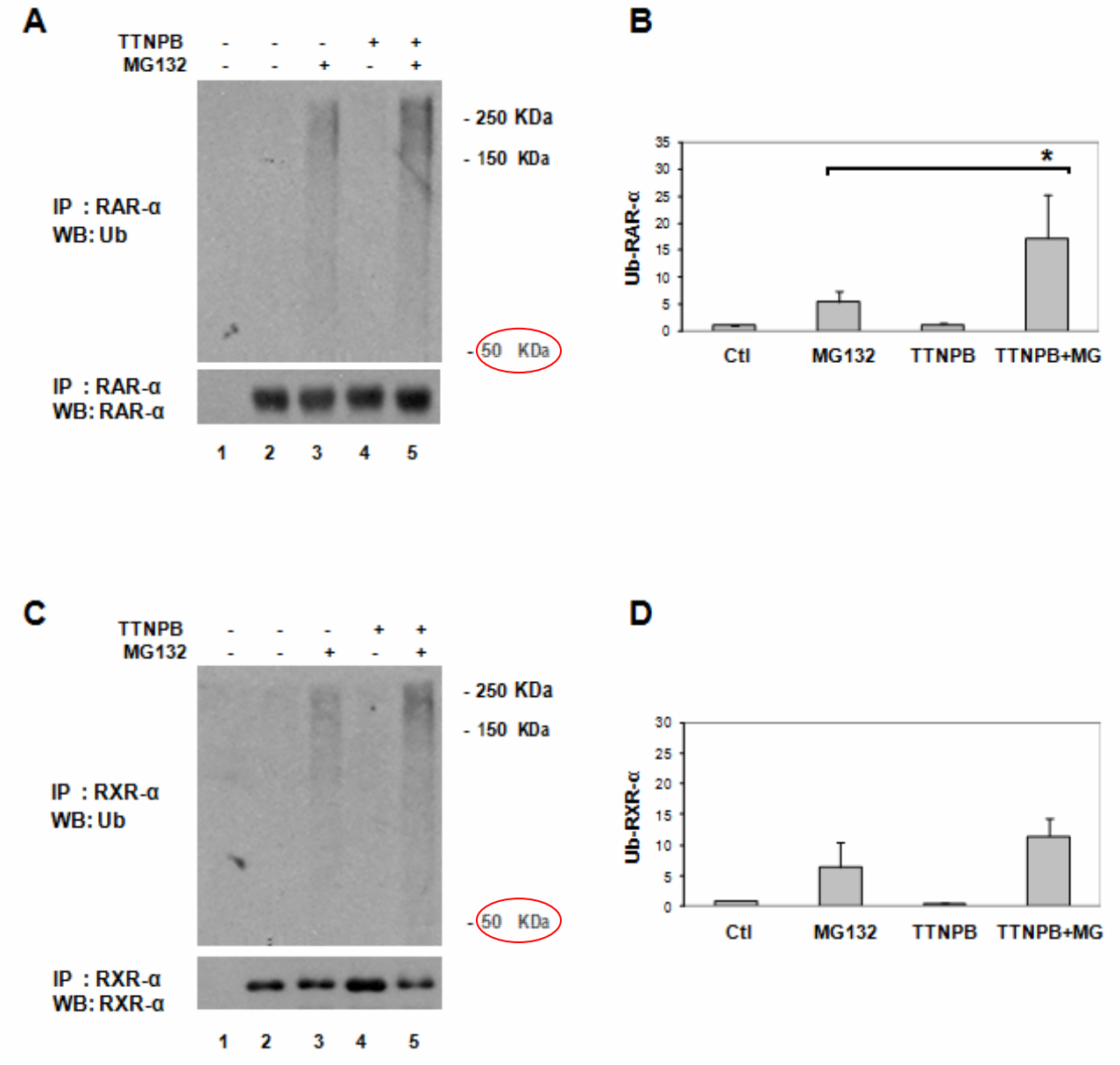

A
TTNPB - - - + +
MG132 - - + - +
- 250 KDa
- 150 KDa
IP : RAR-α
WB: Ub
- 50 KDa
IP : RAR-α
WB: RAR-α
1 2 3 4 5
B
Ub-RAR-α
35
30
25
20
15
10
5
0
*
Ctl
MG132
TTNPB
TTNPB+MG
C
TTNPB - - - + +
MG132 - - + - +
- 250 KDa
- 150 KDa
IP : RXR-α
WB: Ub
- 50 KDa
IP : RXR-α
WB: RXR-α
1 2 3 4 5
D
Ub-RXR-α
30
25
20
15
10
5
0
Ctl
MG132
TTNPB
TTNPB+MG

Figure 4.12 **Effects of RAR-selective Ligand on retinoid receptors ubiquitination in P19 cells**

(A) Immunoprecipitation of RAR-α in P19 cells treated with MG132 (5 μM) and/or TTNPB (1 μM) for 4 hrs in comparison to untreated control cells. Western blots were first probed with anti ubiquitin antibody then reprobed with anti RAR-α antibodies. The first lane represents control cells without IP. (B) Quantitative analysis of panel A presented as fold variation in comparison to untreated control cells ($*p \leq 0.05$). Error bars represent the standard deviation of three independent experiments. (C) & (D) The same as described in A and B but RXR-α was immunoprecipitated. The red circles in panels A and C indicate where RAR-α and RXR-α were migrated.

4.13 Inhibition of the 26S proteasome enhances the colocalization of ubiquitin with Ligand-induced RAR-α in cytoplasmic foci

To determine if the ligand-activated RARs-α in the cytoplasmic foci and the nuclear microfoci are ubiquitinated or not, we examined the colocalization of RAR-α with ubiquitin protein by immunofluorescent staining. Immunofluorescent staining was done to visualize P19 cells treated for 4 hours with RAR-specific ligand TTNPB and/or with proteasome inhibitor MG132 using antibodies against RAR-α and ubiquitin plus fluorescent labeled secondary antibodies. ApoTome optical sectioning was performed and the cells were visualized by immunofluorescent microscopy. We found that ubiquitins were colocalized with ligand-bound RARs-α in the pre-nuclear cytoplasmic foci of concomitantly treated cells. As well, the ubiquitins were colocalized with RARs-α within nuclear microfoci in cells only induced with RAR-specific ligand TTNPB without proteasomal inhibition (fig. 4.13). These data add more evidence to confirm the ubiquitination of liganded RAR-α but if this ubiquitination of RAR-α is for their degradation or not is not clear.

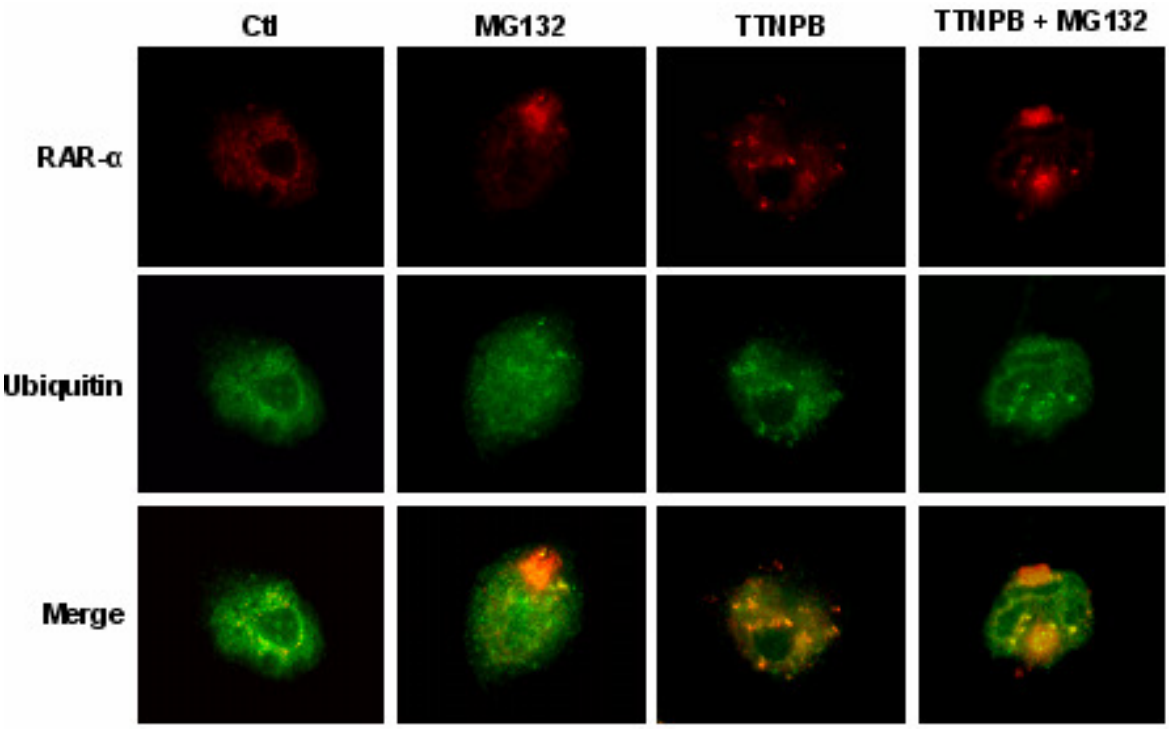


Figure 4.13 **Inhibition of the 26S proteasome enhances the colocalization of ubiquitin with Ligand-induced RAR-α in cytoplasmic foci**

Immunofluorescence microscopy with ApoTome optical sectioning was used to visualize P19 cells treated with TTNPB (1 μM) and/or with MG132 (5 μM) for 4 hrs, fixed, and then stained with anti RAR-α (shown on red) and anti- ubiquitin (shown on green) antibodies followed by secondary staining in comparison to untreated controls. The merged images of both channels are shown in the bottom. Shown are representatives of single tomography. The experiment was repeated at least three times.

4.14 The 20S subunit of the proteasome is not colocalized with RAR-α within short term RAR-specific ligand induction

To assess the impact of RAR-α ubiquitination on its degradation, we first carried out an immunofluorescent staining to colocalize RAR-α with the 20S subunit of the proteasome. P19 cells were grown on cover slips, treated as in fig. 4.10, fixed and then stained with antibodies against RAR-α and α subunits of the 20S proteasome then with secondary antibodies. Immunofluorescent microscopy with ApoTome optical sectioning was used to visualize these cells. We found that the 20S subunit of the proteasome was not colocalized with RAR-α in the perinuclear cytoplasmic foci either under conditions of proteasome inhibition only or plus ligand induction. Also, there was no colocalization of RAR-α and 20S proteasome in the nuclear microfoci that appear with TTNPB ligand induction (fig. 4.14). These data suggest that the ubiquitination of ligand-activated RAR-α after short term TTNPB treatment (4 hours) is for transcriptional regulation of RAR-regulated genes and not for receptor degradation.

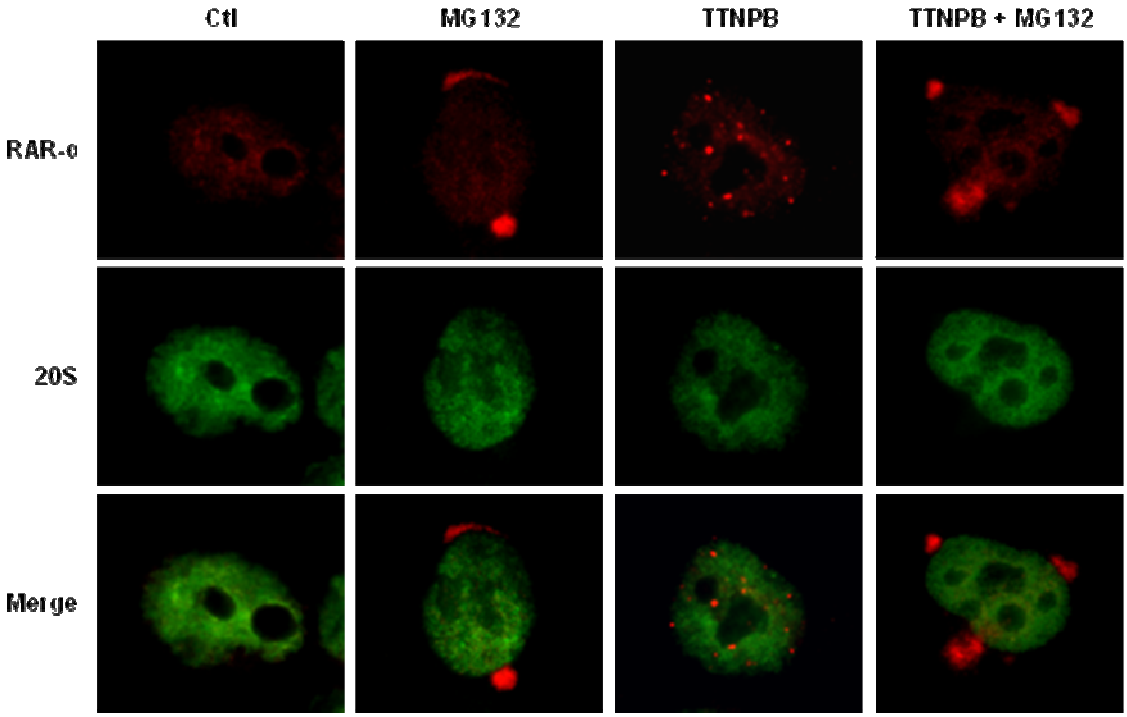


Figure 4.14 **The 20S subunit of the 26S proteasome is not colocalized with liganded RAR-α**

Immunofluorescence staining using antibodies against RAR-α (red channels) and 20S subunit of the 26S proteasome (green channels) was done in P19 cells treated with TTNPB (1 μM) and/or MG132 (5 μM) for 4 hrs in comparison to untreated control cells followed by specific secondary antibodies. ApoTome optical sectioning was performed. The lower panels represent the merge of the two channels. The experiment was repeated at least three times.

4.15 RAR-specific ligand induction has no effects on the stability of both RAR-α and RXR-α in P19 cells

We aimed to further identify if this previously mentioned enhancement of retinoid receptors ubiquitination with RAR-specific ligand treatment is related to the role of ubiquitination in protein turnover or due to its role in transcriptional regulation. Western blot assays were performed to investigate the effects of TTNPB treatment and proteasome inhibition on the turnover of RAR-α, RXR-α, p300, SRC-1 and N-CoR (fig. 4.15 A, B, C & D) in P19 cells treated with RAR-selective ligand TTNPB in the presence or absence of proteasome inhibitor MG132 for 4, 8 and 16 hours. We found that the protein levels of retinoid receptors and their coactivators were not significantly changed within the time course of the assays in cells treated with MG132 (lanes 2, 5 and 8), TTNBP (lanes 3, 6 and 9) or concomitantly treated with both of them (lanes 4, 7 and 10) in comparison to untreated control cells (lane 1) (fig. 4.15 A, B, C & D). However, the protein levels of N-CoR were elevated with proteasome inhibition in cells treated only with proteasome inhibitor either alone (lanes 2 and 5) or with RAR-selective ligand induction (lanes 4 and 7) in comparison to untreated cells (lane 1). This elevation of N-CoR protein levels was observed only at 4 and 8 hours of treatment but not at 16 hours (fig. 4.15 C & D).

Next, pulse chase experiments were performed using S^{35} labeled methionine/cysteine to pulse P19 cells for 4 hours before chasing and inducing them with TTNPB for 4, 8, 16 and 24 hours. Then RAR-α was immunoprecipitated and the remaining radioactivity was measured (fig. 4.16 A). Additionally, Western blot assays were done using 10 µg/ml of the protein synthesis inhibitor cycloheximide (Baliga et al.,

1969) and/or TTNPB for 4, 8, 16 and 24 hours to measure RXR-α protein levels (fig. 4.16 B). The half-life of both receptors was measured accordingly and it was shown that TTNPB ligand induction does not affect the stability of both RAR-α and RXR-α as show in tables (fig. 4.16 A & B).

All together, these data suggest that the RAR-specific ligand-dependent ubiquitination of retinoid receptors has no effects on the stability of both RAR-α and RXR-α. Accordingly, the UPS is involved in regulating RAR-regulated genes through its non proteolytic functions.

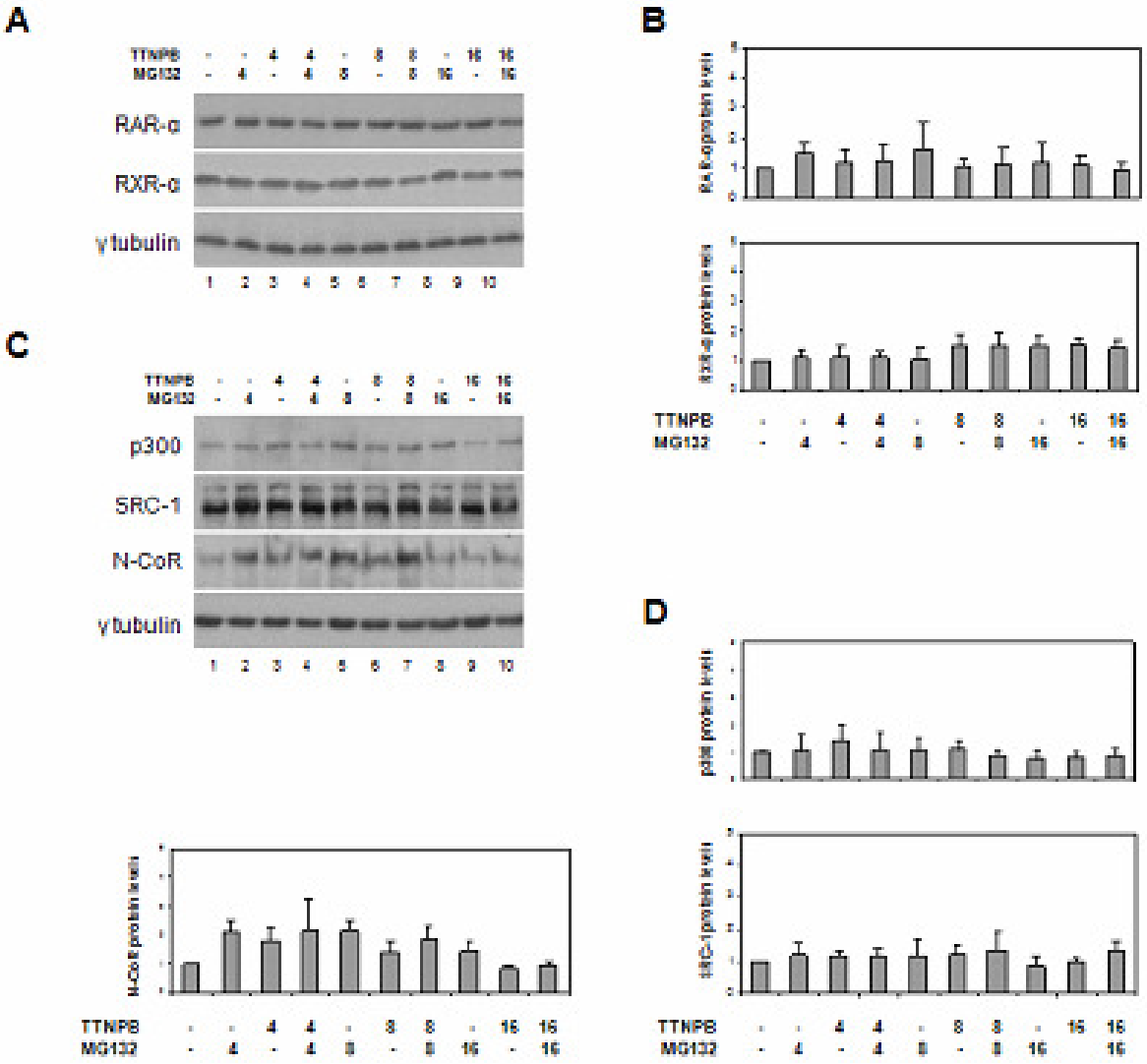
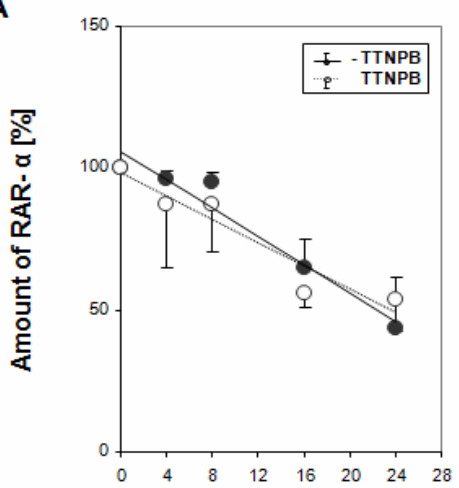

A
TTNPB
MG132
RAR-α
RXR-α
γtubulin
1 2 3 4 5 6 7 8 9 10
B
RAR-α protein level
RXR-α protein level
C
p300
SRC-1
N-CoR
γtubulin
N-CoR protein level
D
p300 protein level
SRC-1protein level

Figure 4.15 **The effects of proteasome inhibition on the protein levels of RAR-α, RXR-α, coactivators and N-CoR within short term RAR-selective ligand induction**

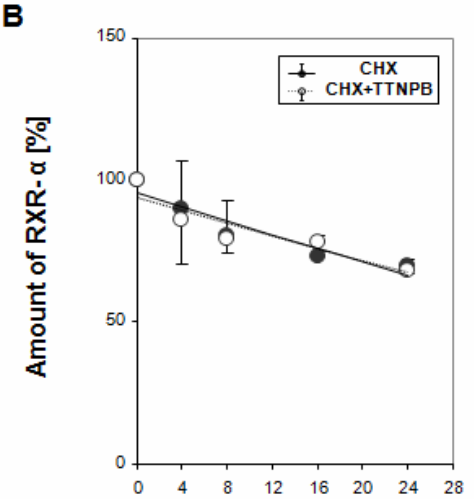
(A) Western blots analysis of the protein levels of RAR-α and RXR-α in P19 cells treated with MG132 (5 μM) and/or TTNPB (1 μM) for 4, 8 and 16 hrs. γ tubulin is the protein loading control. (B) Quantitative analysis of panel A is presented as fold variations compared to untreated cells after being normalized to the loading controls. Error bars represent standard deviations of at least five independent experiments. (C) Western blots analysis of equal amounts of protein from P19 cells whole cell extract (50 μg) to detect the protein levels of p300, SRC-1 and N-CoR following treatments with MG132 (5 μM) and/or TTNPB (1 μM) for 4, 8 and 16 hrs. The blots were then reprobed with γ tubulin as internal control. (D) Quantitative analysis of panel C expressed as fold variations in comparison to untreated control after being normalized to the internal controls. Error bars represent standard deviations of at least five independent experiments.

A



	- TTNPB	TTNPB
$T^1/_2$ RAR-α [hrs]	23.31 ± 5.11	24.49 ± 3.65
Correlation Coefficient	- 0.974	- 0.955
R^2	0.95	0.91

B



	CHX	CHX+TTNPB
$T^1/_2$ RXR-α [hrs]	37.38 ± 1.47	40.16 ± 2.91
Correlation Coefficient	- 0.946	- 0.922
R^2	0.895	0.849

Figure 4.16 **The effects of RAR-α ligand-induction on the half lives of both RAR-α and RXR-α in P19 cells**

(A) Quantitative analysis of immunoprecipitated RAR-α protein levels in P19 cells pulsed for 4hrs then chased and treated with TTNPB (1 μM) for 4, 8, 16 and 24 hrs in comparison to untreated (no chase) control cells or harvested at the same time points but with no treatment in comparison to untreated (no chase) cells. Values are presented as mean ± standard deviation of the percentage of remained radioactivity in comparison to no chase cells remaining radioactivity. (B) Quantitative analysis of RXR-α protein levels in P19 cells treated with Cycloheximide (CHX 10 μg/ml) ± TTNPB (1 μM) for 4, 8, 16 and 24 hrs after performing Western blots. Values are presented as mean ± standard deviation of the percentage of protein levels in comparison to untreated control cells. The 2 tables are representatives of the half life ($T^1/_2$) of both RAR-α and RXR-α proteins as detected from pulse chase and protein synthesis inhibition experiments. Both pulse chase and Western blot experiments were repeated at least three times.

CHAPTER 5: DISCUSSION, SUMMARY AND SIGNIFICANCES

5.1 Discussion

Retinoid receptors regulate the expression of a wide array of genes which exert pivotal functions in many physiological processes upon their stimulation by retinoids (Gillespie and Gudas, 2007a). The activity of retinoid receptors themselves and hence, of their regulated genes needs to be tightly controlled at multiple levels to ensure their adequate and timely ordered transactivation [reviewed in (Chambon, 1996)]. Numerous control mechanisms seem to have evolved [(Bour et al., 2007; De los Santos et al., 2007; Gaillard et al., 2006; Perissi et al., 2004) and reviewed in (Bastien and Rochette-Egly, 2004; Xu et al., 1999a)]. In our study, we examined the roles of the UPS in this control process.

It has been reported that the function of the UPS is essential for transactivating RA-responsive genes (Perissi et al., 2004) through unidentified mechanisms. RARs and RXRs are spontaneously expressed in many cells including F9 and P19 mouse EC cells as well as different leukemia cell lines and other cancer cell lines (Boylan et al., 1995; Brooks et al., 1996; Chen et al., 1996; Gianni et al., 1996; Giannini et al., 1997; Taneja et al., 1996). In this thesis, we used P19 cells to address our objectives. RAR-α subtype of retinoid receptors is predominantly expressed in P19 cells, while RAR-β is induced in these cells immediately following RA activation (Giannini et al., 1997; Pratt et al., 2000; Taneja et al., 1996). Additionally, an early induction of other numerous direct RA target genes such as Cyp26A1 has been shown in these cells (Vucetic et al., 2008). Our data demonstrate that the 26S proteasome serves as an activator for RA target genes via

regulatory mechanisms other than its proteolytic function. Additionally, we showed that proteasome activity and ligand induction are involved in the subcellular localization and ubiquitination of retinoid receptors, respectively. All together, these findings provide a novel insight into the regulatory signals of RAR-α/RXR-α-regulated genes through the 26S proteasome. In addition, we highlighted in the current thesis some functional roles of ubiquitination in modifying retinoid receptors and, therefore, their activated genes.

5.1.1 The 26S proteasome serves as an activator for direct retinoic acid target genes

The requirement of the 26S proteasome function (proteolytic or non proteolytic) for activating RAR-regulated genes as well as genes regulated by other nuclear receptors, such as estrogen, androgen, progesterone and thyroid hormone receptors, is well established (Dennis et al., 2005; Lin et al., 2002; Lonard et al., 2000; Perissi et al., 2004). However, a clear understanding of a mechanistic role for the 26S proteasome in transactivating RA-responsive genes is still missing. The key issue addressed in our study was to recognize the molecular mechanisms by which the 26S proteasome transactivates RAR-regulated genes. We discovered here that the activity of the proteasome is fundamental for maintaining the occupancy of RAREs by liganded RAR-α/RXR-α heterodimers and hence by their coactivators such as SRC-1 and p300 (fig.4.2 B & C, fig. 4.3 B, fig.4.4 C & D, fig. 4.5 and fig. 4.6 C & D). Likewise, it was revealed before that the cyclic increase in the occupancy of ligand-activated luteinizing hormone (LH)-β promoters by both Egr-1 and SF-1 in LβT2 gonadotrope cells is proteasome-dependent (Walsh and Shupnik, 2009). As well, the recruitment of phosphorylated RNA

Pol II to these promoters was shown to be inhibited by proteasome inhibitors (Walsh and Shupnik, 2009). In contrast, it was reported that the use of the proteasome inhibitor, MG132 for treating human prostate carcinoma cells in the presence of androgen prevents the release of androgen receptors from prostate-specific antigen promoters (Kang et al., 2002). All together, these reports, in addition to ours, indicate that the function of the 26S proteasome is involved in promoter occupancy either positively or negatively. However, in our study we discovered for the first time that the proteasome-dependent occupancy of ligand-activated RAREs is restricted to RAREs which are in close proximity to the TATA box (fig.4.4 C & D, fig. 4.5 and fig. 4.6 C & D).

Furthermore, a significant increase in the protein levels of the retinoid receptors corepressor, N-CoR, following proteasome inhibition was shown in our study (fig. 4.15 C & D). This finding is in agreement with previous reports that indicated that N-CoR is a target for UPS mediated degradation (Zhang et al., 1998). Moreover, this finding along with the unchanged protein levels of RAR-α, RXR-α, SRC-1 and p300 with proteasome inhibition verify that the repressive effects of proteasome inhibition on RAR-β gene expression are not due to general cellular toxicity or to a global block in the transcriptional ability of these cells. Despite this revealed failure of N-CoR degradation in our system with proteasome inhibition, we did not demonstrate any association between N-CoR and liganded RAR-α/RXR-α (fig. 4.9 and data not shown). As well, we did not find any occupancy of RARE by this corepressor with proteasome inhibition of ligand-induced cells (fig. 4.2 B & C). Therefore, we cannot attribute the repressive effect of proteasome inhibition on RAR-β2 gene expression to persistence of retinoid

receptor/N-CoR association which -if present- will lead to an inability of coactivators binding to RAR/RXR.

At this point, two questions remain to be answered: **1)** what are the mechanisms that allow RAR-α/RXR-α to be removed from their proximal DNA responsive promoters; and **2)** what is the molecular basis of the variation between R1 and R2 response elements regarding their occupancy by liganded retinoid receptors heterodimers upon proteasome inhibition? There is a possibility that the answer to these questions could be tightly linked. This means that there must be a mediator protein in the vicinity of TATA box that is modulated by the 26S proteasome and also involved in preserving the binding of liganded RAR/RXR to RAREs. The proteasome inhibition-dependent loss of the function of this protein with ligand activation and/or the failure of its recruitment to the R1 region may result in the demonstrated loss of the occupancy of the R1 but not the R2 regions by activated retinoid receptors complexes. For instance, it has been shown that activation of p38MAPK/MSK1 phosphorylation pathway upon RA treatment increases the recruitment of RAR-α/TFIIH complexes to RARE (Bruck et al., 2009). Moreover, it was shown that there is occupancy of the R1 region by the XPB, cdk7 and cyclin H subunits of the TFIIH without ligand but not of R2 (Bruck et al., 2009). This occupancy was shown to be increased with ligand induction in both regions but this increase was about two folds more with the R1 than the R2 regions (Bruck et al., 2009). Accordingly, differences were shown between the binding of TFIIH to the R1 and R2 regions. TFIIH is a general transcription factor that encompasses cyclin activating kinase-subcomplexes including cdk7 and cyclin H and occupies the TATA box region along with other general transcription factors and RNA Pol II (Lee and Young, 2000). This factor is a

downstream target for MSK1 in the p38MAPK/MSK1 phosphorylation pathway (Bruck et al., 2009). TFIIH induces phosphorylation of RAR-α at serine 77 which in turn enhances RAR-α/TFIIH association and hence their DNA binding (Bruck et al., 2009). Moreover, optimum phosphorylation levels were reported to be essential for maintaining the DNA binding of many nuclear receptors including retinoid receptors (Gaillard et al., 2006; Hirata et al., 1993; Katz et al., 1995). Thus, we hypothesized that the expression of this transcription factor as well as its binding to the R1 region may be decreased by proteasome inhibition. This results in a decrease in the phosphorylation of RAR-α within the R1 region only. Therefore, a decrease in the binding affinity of RAR-α to the R1 region may occur. Nevertheless, our data show no change in the levels of phosphorylated serines within RARs-α between TTNPB induced cells and cells activated by TTNPB with proteasome inhibitors through immunoprecipitation assays (data not shown). Additionally, we found no differences between the R1 and R2 regions regarding their occupancy by TFIIH (fig. 4.7 B). Also, the occupancy of both R1 and R2 regions by RNA Pol II as a protein that occupies the TATA box was studied in our system (fig. 4.7 A). Again, no proteasome-dependent differences were demonstrated between the occupancy of R1 and R2 region by this protein in the presence or absence of RAR-specific ligand. This indifference between R1 and R2 regions regarding their occupancy by RNA Pol II with ligand activation is similar to what shown before in the Bruck et al. 2009 study (Bruck et al., 2009).

Further studies would be required in the future to elucidate the regulatory signals of retinoid receptor/DNA binding that could explain these demonstrated differences between the R1 and R2 regions. This could be conducted through mass spectrometry to

characterize the proteins which occupy both basal and ligand-activated RA-responsive promoters that include a TATA box e.g. R1 region. Classifying the resultant proteins then performing ChIP assays using antibodies against these proteins could be done later to validate the results. Moreover, ChIP-seq using anti RAR-α and RXR-α antibodies may be carried out followed by bioinformatics analysis to identify the genome wide effects of proteasome inhibition on the occupancy of RAREs by liganded retinoid receptors.

Also, our data show that DNA-free RAR-α is associated with DNA-free RXR-α only in the presence of ligand which matches with what was reported before in Depoix et al. study (Depoix et al., 2001). Likewise, thyroid hormone has been shown to enhance the heterodimerization between thyroid hormone receptors and RXRs in solution both *in vivo* and *in vitro* (Collingwood et al., 1997). However, we demonstrated here for the first time that this association between liganded RAR-α and RXR-α in solution is proteasome-dependent as well. Consequently, the activity of the 26S proteasome is crucial for DNA-independent interaction between endogenous retinoid receptors and their coactivators in response to ligand induction. All together, our study reveals that the inhibition of the 26S proteasome results in reduction of the DNA-independent RAR/RXR heterodimerization upon ligand binding. This heterodimerization was shown before to be crucial for retinoid receptor binding to DNA and hence, for transactivating RA-responsive genes (Depoix et al., 2001). Thus, this finding may provide an earlier level for controlling retinoid signaling by the 26S proteasome even before binding of liganded RAR/RXR to DNA response elements.

To summarize this section, we can conclude that the non-proteolytic function of the proteasome is a key regulator for RA-responsive genes. This regulatory function is

required at early level following ligand activation for inducing the DNA-independent interaction between RAR-α and RXR-α and hence for maintaining the occupancy of retinoid-responsive promoters by liganded RAR/RXR heterodimers. To my knowledge, this is the first time the involvement of the 26S proteasome activity in receptors heterodimerization is addressed. Accordingly, these findings open new avenues of deliberation that could be considered in future research regarding the mechanisms mediating the proteasome function in regulating the expression of other genes.

5.1.2 The regulatory role of the 26S proteasome in the subcellular localization of RAR-α and RXR-α within P19 cell line

RAR-α belongs to the same subfamily of nuclear receptors as thyroid hormone/vitamin D receptors. Members from this nuclear receptor subfamily were thought before to be localized merely in the nucleus either in the resting (no ligand activation) or active states [reviewed in (Mangelsdorf et al., 1995; Tsai and O'Malley, 1994)]. We have shown here that the subcellular localization of RAR-α is not restricted to the nucleus but could be cytoplasmic as well. In our study, RAR-α was visualized within perinuclear cytoplasmic foci only following proteasomal inhibition. RXR-α as well as retinoid receptors coactivators, including SRC-1 and p300, were shown to be localized constitutively in the nuclei. It is important to report that this cytoplasmic localization of RAR-α was not specific to a certain antibody because two different anti-RAR-α antibodies were used (anti RAR-α "C20" and anti RAR-α MAb) and both gave the same results. All together, these results suggest that the subcellular distribution of RAR-α but not RXR-α in P19 cells is proteasome-dependent. Therefore, the proteasome

may be considered as a complex that could be involved in controlling the subcellular localization of other nuclear receptors.

The possibility of cytoplasmic localization of retinoid receptors is in accordance with a number of former studies. For example, it has been reported that decreased expression of protein kinase C enhances the cytoplasmic localization of RAR-α in COS-7 cells (Tahayato et al., 1993). Furthermore, RAR-α was found in the cytoplasm of both Sertoli cells in young animals (Dufour and Kim, 1999) and germ cells with vitamin A deficiency (Akmal et al., 1998). In a previous study using immunoelectron microscopy, RAR-γ was shown to accumulate around the rough endoplasmic reticulum in endometrial epithelial cells during the secretory phase of the menstrual cycle (Fukunaka et al., 2001). It is equally important to note that the promyelocyte-RAR-α fusion oncoprotein (PML-RAR-α) was observed before within cytoplasmic compartments and was translocated to the nucleus with RA treatment in acute promyelocytic leukemia (APL) (Weis et al., 1994). Moreover, in another study it has been recognized that a small ubiquitin-like modifier-2 (SUMO-2) protein mediates post translational modifications of RAR-α which is essential for controlling the ligand-dependent subcellular trafficking of these receptors in Sertoli and COS-7 cells (Zhu et al., 2009). Similarly, other members from the same subfamily of nuclear retinoid receptors were demonstrated in the cytoplasm of different cells. These members include thyroid hormone receptors that were shown to have ligand-dependent nuclear/cytoplasmic trafficking and PPAR-α which was found mainly in cytoplasmic compartments (Chinetti et al., 1998; Zhu et al., 1998). Considering all of this evidence, we can conclude that RAR-α may be located in both the nucleus and the

cytoplasm and this is regulated by diverse factors and differs from one cell line to another.

The mechanisms by which retinoid receptors reside in the cytoplasm are not well characterized although different mechanisms may be postulated (Zhu et al., 1999b). For instance, a defect in RAR-α nuclear retention resulting from its hyperactive nuclear export may be involved in our study. This could be enhanced by the detected decline in the DNA binding capacity of liganded receptors with proteasome inhibition. We can hypothesize that this proposed hyperactive nuclear export may result from accumulation of nuclear RAR-α carrier proteins after inhibition of the 26S proteasome as these proteins may be targets for proteasomal degradation. These proteins may be mainly in charge of transferring RAR-α from the nucleus to the cytoplasm. However, these proteins are still not identified and may be a future direction of interest. Conversely, failure in the transport of RAR-α from the cytoplasm to the nucleus may also be postulated. Thus, RAR-α resides predominantly in the cytoplasm. In turn, this may participate in explaining the effects of proteasome inhibition in repressing RA-responsive genes as RAR-α, by this way, is incapable of accessing or binding to RAREs and hence incapable of transactivating its target genes. Also, the ligand-dependent ubiquitination of RAR-α which was demonstrated in the current study may play a mechanistic role in RAR-α subcellular localization specially because ubiquitination *per se* was shown before in many reports to be involved in controlling the subcellular trafficking of a number of proteins such as HTLV-I oncoprotein Tax (Gatza et al., 2007) and DNA Pol eta and iota (Plosky et al., 2006). To conclude, we can say that RAR-α subcellular trafficking is a

highly complex process along with its mechanistic pathways and thus further studies will be required to elucidate them.

Importantly, our results clearly indicate that the cytoplasmic localization of RAR-α in P19 cells that has been shown here is not due to absence of ligand, as suggested in previous studies (Yamashita, 1998). Indeed, cytoplasmic foci are visualized with proteasomal inhibition of both liganded and unliganded RAR-α. Moreover, we found that the number of these foci per cell is higher with proteasomal inhibition of ligand-induced cells than of inactivated cells. This could be due to the proteasome inhibition-dependent decrease in the binding of RAR-α to their RARE. Therefore, more free RAR-α will be available for cytoplasmic translocation. As well, the enhanced ubiquitination of RAR-α with ligand activation could be involved in this ligand-dependent increase in foci numbers per cell following proteasome inhibition. This idea is supported here by the colocalization of ubiquitin protein with liganded RAR-α that we have observed within these foci (fig. 4.13). It is also important to mention that this elevated number of foci per cell with concomitant ligand induction and proteasome inhibition is not caused by an increase in the synthesis of RAR-α with ligand activation. This is because RAR-specific ligand activation in our study did not elevate either the retinoid receptors protein levels or their binding to DNA as indicated from Western blots and ChIP assays respectively (fig. 4.15 A & B, fig. 4.2 B & C, fig. 4.4 C and fig. 4.6 C). Ligand induction in our study led to accumulation of RAR-α primarily within intra-nuclear microfoci which may point to an enhancement of receptor-DNA binding with ligand activation. However, ChIP assays in our study did not show any significant increase in the occupancy of RAREs by RARs-α with RAR-specific ligand induction (fig. 4.2 B & C, fig. 4.4 C and fig. 4.6 C). A

possible reason for this could be an oscillation in the binding of RAR/RXR heterodimers to DNA because of ligand-induced transcriptional cycles to supply RAREs with fresh receptors. Therefore, an increase in the occupancy of RAREs by liganded receptors may be replaced in our system by their ligand-dependent cyclic recruitment to these responsive regions.

The ubiquitin/proteasome system was shown to be integrated in many cytoplasmic and nuclear inclusion bodies which are hallmarks of numerous neurodegenerative diseases such as Parkinson and Huntington diseases (McNaught and Jenner, 2001; Sherman and Goldberg, 2001). However, in our study neither cytoplasmic foci nor intra-nuclear microfoci were shown to be colocalized with the 20S proteasome (fig. 4.14). Intra-nuclear microfoci as well as cytoplasmic foci that contain liganded RAR-α were revealed here to be associated with ubiquitin proteins (fig. 4.13). These findings provide additional support to our conclusion which states that short term induction by RAR-specific ligand enhances RAR-α ubiquitination but not turnover in P19 cells.

5.1.3 RAR-specific ligand and ubiquitination of RAR-α/RXR-α in P19 cells

In the current study, we investigated ligand-mediated RAR-α/RXR-α ubiquitination in P19 cells and further inquired into their resultant influences on receptor turnover. Our findings reveal that RAR-specific ligand significantly enhances the ubiquitination of RAR-α more than three fold compared to unliganded RAR-α whereas, in the case of RXR-α, this induction of ubiquitination was less than two fold. Thus, RAR-specific ligand enhances distinct levels of ubiquitination between RAR-α and RXR-

α. Consistent with our findings, the ubiquitination of retinoid receptors upon ligand induction has been revealed in earlier studies (Ferry et al., 2009; Gianni et al., 2002; vom Baur et al., 1996; Wu et al., 2004). However, what is really intriguing in our findings is that the ubiquitination here was not accompanied by receptor degradation as indicated in previous studies. By taking these results into consideration, we can propose that the ligand-dependent ubiquitination of RAR-α and RXR-α in P19 cells is not for their degradation. This is supported by the insignificant changes that were detected in our report in the half lives of both RAR-α and RXR-α following TTNPB ligand activation.

Through *in vivo* ubiquitination assays in our study, higher molecular mass ubiquitin complexes that appear as intense and extended bands within the range of (150 -250 kDa) were demonstrated to be associated with RAR-α and RXR-α. However, the molecular mass of ubiquitin protein is about 8.6 kDa (Weissman, 2001). Thus, multiple ubiquitin proteins are covalently attached to the 50 kDa RAR-α and 54 kDa RXR-α. An additional ubiquitination of other proteins that are attached to these endogenous retinoid receptors could be suggested as well and hence causes these higher bands of ubiquitin retinoid receptors complexes. These proteins may be within the coregulator complex that bound to these receptors. Nevertheless, the coactivators p300 and SRC-1 are ruled out from this ubiquitination band because the coimmunoprecipitation experiments shown in fig. 4.8 and fig. 4.9 reveal that these coactivators are not linked with either liganded RAR-α or liganded RXR-α upon proteasomal inhibition. Previously, similar higher molecular mass ubiquitin complexes that are attached to RAR-α and RXR-α have been reported (Adachi et al., 2002; Boudjelal et al., 2000; Srinivas et al., 2005; Wu et al., 2004). To confirm the effects of RAR ligand activation on the ubiquitination of RAR-α and RXR-α,

immunoprecipitation of endogenous or His-tagged ubiquitins, followed by detection of their conjugated RAR-α or RXR-α could be performed. Moreover, a more precise mass spectrometer may be used to further verify our conclusion and to assist in identifying the number of ubiquitins attached to these retinoid receptors, their other uncharacterized post translational modifications, the possibility of novel ligand-dependent coregulators binding to these receptors and to determine if these coregulators are ubiquitinated or not.

Coming to this point, the interesting question will be about the mechanisms underlying the unchanged stability of liganded RAR-α in P19 cells despite their ubiquitination. This unchanged stability suggests that these ubiquitinated retinoid receptors did not bind to the proteasome which leads us back to the previously reviewed literature in the introduction. These studies conclude that not all ubiquitinated proteins are degraded and the character of ubiquitin chain itself determines the fate of its bound protein substrate [reviewed in (Conaway et al., 2002; Muratani and Tansey, 2003)]. Therefore, future research could be directed toward determining the identity of the regulators of this ubiquitination process, recognizing the type or characters of this ubiquitination as well as classifying the functional consequences of this ubiquitination on retinoid receptors and hence on their regulated genes. Moreover, it was reported before that the lysine residues within the AF1 region of RXR-α, RAR-α and γ2 are ubiquitinated but this ubiquitination triggers degradation of these receptors via the ubiquitin/proteasome system (Ferry et al., 2009; Gianni et al., 2002; vom Baur et al., 1996). This means that this RAR-α region may not be the one ubiquitinated in our system. Thus, special attention is needed in the future toward determining which domain structure within RAR-α and RXR-α mediates this reported ubiquitination, which does not

target these receptors for degradation, and toward the identification of the E2 and E3 ubiquitination enzymes that direct this ubiquitination.

5.1.4 The unique behavior of RAR-specific ligand in P19 cells toward RAR-α turnover

The 26S proteasome is the major cellular machinery responsible for degrading a number of nuclear receptors following their ligand binding including RXRs, RAR-α and γ (Hauser et al., 2000; Lange et al., 2000; Lonard et al., 2000; Tanaka et al., 2001b; Zhu et al., 1999a). In a previous report, RAR-γ proteolysis via the ubiquitin/proteasome system was demonstrated after 24 hours of induction by at-RA (10^{-6} M) whereas RAR-α degradation was visible as early as 3 hours following activation by the same ligand and with the same concentration in MCF-7, human breast cancer cells. Furthermore, concentration- and time-dependent effects of at-RA on degradation of these receptors were observed as well (Tanaka et al., 2001b). In contrast, Zhu et al. in 1999 found that RAR-α proteolysis in NB4 cells (human acute promyelocytic leukemia cells) was not clearly observed before 12 hours of at-RA (10^{-6} M) induction. Moreover, they reported that the RAR-specific ligand, TTNPB (10^{-6} M) activation induced the degradation of PML-RAR-α 24 to 48 hours later than at-RA (10^{-6} M)-treatment (Zhu et al., 1999a). Additionally, at-RA was shown in the same study to induce RAR-α degradation in U937 and HL-60 leukemia cells in a concentration-dependent manner. This degradation appears only following overnight treatment of these cells with at-RA (Zhu et al., 1999a). In the current thesis, we investigated the implications of a RAR-specific ligand (TTNPB) induction on the stability or turnover of both RAR-α and RXR-α. Interestingly unlike the

previous reports, we demonstrated that short term ligand induction (4-16 hours) by TTNPB had no effect on the stability of both RAR-α and RXR-α. Also, a 50% reduction in the protein levels of RAR-α was detected in our study after 24 hours of TTNPB induction. Moreover, RXR-α half life was detected in this thesis after more than 36 hours of RAR-specific ligand induction. Our data together with other reports suggest that variation in cell type plus differences in RA derivatives, concentration, and duration of induction may be responsible for these reported dissimilarities in RAR degradation patterns.

In parallel with our findings, vitamin D and PPAR-δ ligand were reported to inhibit the ubiquitin/proteasome system-dependent degradation of vitamin D3 receptors and PPAR-δ respectively. Thus, these receptors are stabilized with their own ligands (Genini and Catapano, 2007; Li et al., 1999). These two reports in addition to ours imply that nuclear receptor activation by ligand does not strictly associate with their down-regulation as shown before with numerous nuclear receptors (Dennis et al., 2005). A possible suggestion could be that stabilization of retinoid receptors with ligand induction may be required to maintain the transactivation of its regulated genes which would be blocked early if rapid degradation of these receptors happens. Furthermore, our data indicates that the ubiquitin/proteasome system-dependent proteolysis of retinoid receptors is not involved in the early regulatory role of the proteasome in transactivating RAR-regulated genes, at least in our system.

All together, the above data reveal a novel mechanism for the regulation of retinoid signaling through ligand and ubiquitin/proteasome system uncoupled to proteasome mediated degradation of retinoid receptors. The augmented RAR-α ubiquitination with

ligand, the proteasome inhibition-dependent translocation of liganded RAR-α from the nucleus to the cytoplasm beside the dissociation of ligand-bound RAR-α from RXR-α and hence from RAREs are likely to explain why RAR-α is incapable of exerting its function in regulating RA-responsive genes. Though, these demonstrated dissimilarities in the effects of proteasome inhibition between RAR-α and RXR-α regarding both ubiquitination and subcellular localization may reflect specific functions of RAR-specific ligand TTNPB in retinoic acid signaling.

5.1.5 RAR-specific ligand (TTNPB) versus retinoic acid (RA)

The differences in the levels of ubiquitination as well as subcellular localization between RAR-α and RXR-α in P19 cells upon ligand induction or proteasome inhibition with ligand induction may be attributed to the use of TTNPB which is a RAR-specific ligand. This strengthens the hypothesis which suggests that RAR-specific ligands could have a selective role in modulating RARs *per se* but not RXRs. TTNPB is a synthetic derivative of retinoids that binds only RARs (Pignatello et al., 1997) whereas at-RA is a natural retinoid that could be metabolized *in vivo* to 9-cis RA which binds both RARs and RXRs (Germain et al., 2006). Different studies had compared the effects of TTNPB to those of RA regarding gene transcription, cell growth and differentiation in many cell lines. For instance, RA was shown to be more potent than TTNPB in inducing the differentiation of acute promyelocytic (HL-60) cells though they have equal effects on the cell growth and differentiation of acute myelomonocytic leukemia (LK) cells (Fabian et al., 1987). Also, TTNPB was revealed to be more active than RA in promoting the growth of blood cell progenitors from normal human bone marrow cells (Fabian and Shvartzmayer, 1986).

Thus, it is obvious that each specific RA ligand has diverse consequences on cellular processes mediated by RA-responsive genes which are cell type-specific as well. This in turn may explain many of the variations that were demonstrated between our system and other studies where RA was used. We hope that our findings may have significance in improving the potential therapeutics of retinoids because the challenging area of interest recently is directed toward designing retinoids with affinity to specific subtypes of retinoid receptors (Hembree et al., 1996).

5.2 Summary and Significances

In the present study, we demonstrated the diverse regulatory mechanisms underlying the role of the ubiquitin/proteasome system as well as acetylation in transactivating RAR-α-regulated genes. Our data suggest that the non-proteolytic function of the proteasome is required at early level for inducing the association between liganded RAR-α and RXR-α. Moreover, the proteasome activity is crucial for preserving the occupancy of RAREs in the vicinity of TATA box by liganded retinoid receptors/coactivators complex (fig. 5.1 B). We further showed that RAR-α shuttles between the nucleus and the cytoplasm within P19 cells in a proteasome-dependent manner. Thus, the activity of the 26S proteasome is fundamental for regulating the subcellular localization of RAR-α in P19 cells and for maintaining their nuclear localization (fig. 5.1 C). Additionally, we found that RAR-specific ligand activation increases the ubiquitination but not the turnover of both RAR-α and RXR-α in P19 cells (fig. 5.1 D).

We believe that characterizing the diverse mechanisms that modulate the complex retinoid signaling pathways may help in understanding diverse functions attributed to RAR-regulated genes such as developmental processes. Furthermore, this may indirectly contribute in improving the therapeutic applications or the clinical efficacy of retinoids. For example, the combination between multiple cancer chemotherapeutic agents is now increasingly sought in many clinical trials [reviewed in (Garman et al., 2007)]. Since both proteasome inhibitors and retinoids are potential cancer therapeutics, their combined treatment may develop resistant to retinoids therapy in some cancer cells according to what was shown in our system. Therefore, clinicians should be careful when using

proteasome inhibitors in cancer therapy in combination with retinoids. They may even avoid this combination in some cancers to overcome resistance to retinoids. Lastly, developing a new non-toxic synthetic retinoids such as RAR-specific agents that resemble TTNPB in its effects on retinoid receptors stability could be helpful. The use of these agents for treating cancers or other retinoid-responsive pathological conditions such as dermatological diseases [reviewed in (Orfanos et al., 1997; Seaton, 2006)] may result in sustained and prolonged activation of retinoid receptors and thus of their regulated genes.

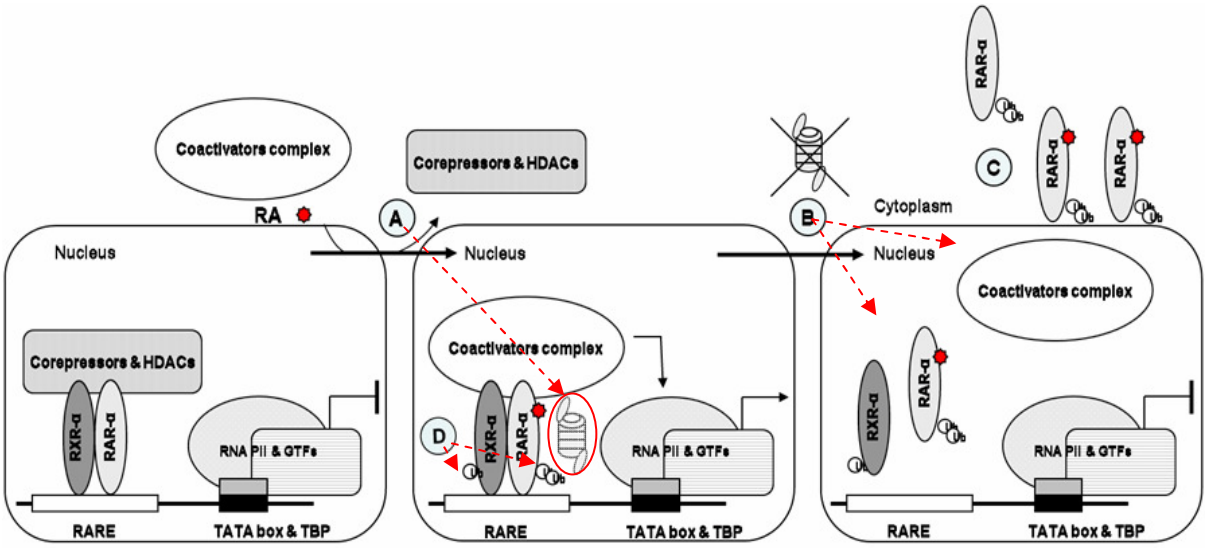


Figure 5.1 **Role of the 26S proteasome and ubiquitination in the regulation of RA-responsive genes**

(A) The 26S proteasome activity is essential for the induction of RA-responsive gene expression. (B) The 26S proteasome activity is crucial for regulating the formation of ligand-induced protein complex that occupies RARE within RA-responsive promoters. (C) Proteasomal inhibition induces the subcellular localization of RAR-α in cytoplasmic foci. (D) Short term induction with RAR-specific ligand enhances the ubiquitination of RAR-α and does not affect its stability.

APPENDIX A: REAGENTS, CHEMICALS AND ANTIBODIES

Table 1.1: **Suppliers of Reagents and Chemicals**

Reagent	**Supplier**
DMEM	Invitrogen -GIBCO (Carlsbad, CA, USA)
methionine/cysteine -free DMEM	Invitrogen-GIBCO (Carlsbad, CA, USA)
Fetal Bovine Serum (FBS)	HyClone (South Logan, UT, USA)
Penicillin/Streptomycin (Pen Strep)	Invitrogen-GIBCO (Carlsbad, CA, USA)
MEM Non-Essential Amino Acids	Invitrogen-GIBCO (Carlsbad, CA, USA)
TTNPB	Sigma-Aldrich (St. Louis, MO, USA)
MG-132	Sigma-Aldrich (St. Louis, MO, USA)
Bortezomib (PS-341)	LC Laboratory (Woburn, MA, USA)
Cycloheximide (CXH)	LC Laboratory (Woburn, MA, USA)
ExGen 500	MBI Fermentas (Glen Burnie, MD, USA)
Luciferase Assay System Kit	Promega-Fisher Scientific (Nepean, ON, Canada)
Total RNA Kit I	Omega Bio-Tek (Norcross, GA, USA)
RNeasy Mini Kit	Qiagen (Mississauga, ON, Canada)
High Capacity cDNA Archive Kit	Applied Biosystems (ABI) (Foster City, CA, USA)

Power SYBR® Green PCR Master Mix	ABI (Foster City, CA, USA)
TaqMan probes	ABI (Foster City, CA, USA)
Bio-Rad Protein Assay dye reagent concentrate	Bio-Rad (Hercules, CA, USA)
Immun-Blot PVDF membrane	Bio-Rad (Hercules, CA, USA)
Western Lightning ^{TM} Chemiluminescence	Perkin Elmer (Woodbridge, ON, Canada)
Bovine Serum Albumin (BSA)	Invitrogen-GIBCO (Carlsbad, CA, USA)
Protein A-conjugated agarose beads	Sigma-Aldrich (St. Louis, MO, USA)
Formaldehyde	VWR-BDH (Mississauga, ON, Canada)
Complete Protease Inhibitor Cocktail Tablets	Roche (Laval, QC, Canada)
Protein A agarose/Salmon sperm DNA	Millipore (Billerica, MA, USA)
Proteinase K	Roche (Laval, QC, Canada)
QIAquick PCR purification Kit	Qiagen (Mississauga, ON, Canada)
Cycle Pure PCR purification Kit	Omega Bio-Tek (Norcross, GA, USA)
GoTaq® flexi DNA polymerase PCR Kit	Promega-Fisher Scientific (Nepean, ON, Canada)
dNTP Mix	Promega-Fisher Scientific (Nepean, ON, Canada)
Paraformaldehyde	Electron Microscopy (EM) Science (Gibbstown, NJ, USA)
Hoechst 33258 pentahydrate	Invitrogen- Molecular Probes (Carlsbad, CA, USA)
^{35}S labeled methionine/cysteine	Perkin Elmer (Woodbridge, ON, Canada)

Table 1.2: **Antibodies and their Suppliers**

Antibody	**Cat. No.**	**Supplier**
RAR-α (C20)	sc-551	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
RXR-α (D20)	sc-553	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
SRC-1 (M-341)	sc-8995	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
p300 (N-15)	sc-584	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
N-CoR (C20)	sc-1609	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
γ tubulin (GTU-88)	T 6557	Sigma-Aldrich (St. Louis, MO, USA)
RAR-α MAb (Rα10)	MA1-810	Affinity BioReagents (ABR) (Golden, CO, USA)
SRC-1 MAb	MA1-840	ABR (Golden, CO, USA)
p300 (Ab-1)	MS-586-P	Thermo Scientific-Dharmacon (Lafayette, CO, USA)
p300 (C-20)	sc-585	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Pol II (A-10)	sc-17798	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
TFIIH p89 (H-300)	sc-20697	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
RAR-β2 (C19)	sc-552	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Ub (P4D1)	sc-8017	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
20S proteasome α7/α8 (B-4)	sc-166761	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Donkey anti-rabbit IgG HRP-linked	NA9340	GE Healthcare (Baie d'Urfe, QC, Canada)
Sheep anti-mouse IgG HRP-linked	NA931	GE Healthcare (Baie d'Urfe, QC, Canada)
Donkey anti-goat IgG HRP conjugate	V8051	Promega-Fisher Scientific (Nepean, ON, Canada)
Alexa Flour®488 goat anti-mouse IgG	A-11001	Invitrogen-GIBCO (Carlsbad, CA, USA)
Alexa Flour®488 goat anti-rabbit IgG	A-11008	Invitrogen-GIBCO (Carlsbad, CA, USA)
Alexa Flour®594 goat anti-rabbit IgG	A-11012	Invitrogen-GIBCO (Carlsbad, CA, USA)

APPENDIX B: OLIGONUCLEOTIDES AND PRIMERS

All the oligonucleotides and primers that were used in our study are from Integrated DNA Technologies (IDT) (Coralville, IA, USA).

Table 2.1: **DNA sequences of oligonucleotides used for luciferase assays**

Reporter	**Sequence (5'-3')**
βRE3-Luc	agcttaaggGGTTCACCGAAAGTTCActcgcat

Table 2.2: **DNA sequences of primers used for real time RT-PCR**

Gene	**Forward primers (5'-3')**	**Reverse primers (5'-3')**
Mouse RAR-β2	GATCCTGGATTTCTACACCG	CACTGACGCCATAGTGGTA
Mouse Cyp26A1	GAAACATTGCAGATGGTGCTTCAG	CGGCTGAAGGCCTGCATAATCAC
Mouse GAPDH	TCGGTGTGAACGGATTTG	GGTCTCGCTCCTGGAAGA

Table 2.3: **DNA sequences of primers used in ChIP assays**

RARE	**Forward primers (5'-3')**	**Reverse primers (5'-3')**
Mouse RAR-β2	GGGAGTTTTTAAGCGCTGTGAG	GGAGCAGCTCACTTCCTACC
Mouse Cyp26A1-R1	CCCGATCCGCAATTAAAGATGA	CTTTATAAGGCCGCCCAGGTTAC
Mouse Cyp26A1-R2	TTCACTGAGATGTCACGGTCC	TTCCCAATCCTTTAGCCTGA

APPENDIX C: MANUSCRIPT

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Promoter context determines the role of proteasome in ligand-dependent occupancy of retinoic acid responsive elements

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Running title: The 26S proteasome and RARE occupancy

Key words: gene regulation, transcriptional coactivator, nuclear receptor, the 26S proteasome, genomic association

Abstract

Retinoid acid receptors are DNA-binding proteins mediating the biological effects of ligands through transcriptional activation. It is known that the activity of the 26S proteasome is important for nuclear receptor-activated gene transcription. However, the molecular mechanism by which the 26S proteasome participates in this process is not well understood. Here we report that the proteasome activity is essential for ligand-dependent interaction of RAR with its coregulators such as SRC, p300 and RXR. We also determined that the proteasome activity is required for the association of liganded RAR to the genomic DNA, consequently for the recruitment of the coactivator complex to the retinoic acid responsive elements. Moreover, the requirement of proteasome activity for the activator activity of RAR is determined by a promoter context. Our study suggests that the 26S proteasome regulates directly the activity of RAR as an activator.

Introduction

In vertebrates, the proper distribution and metabolism of vitamin A is essential for normal embryonic development and growth (Niederreither and Dolle, 2008). Deficiency in vitamin A during early embryogenesis leads to congenital malformations affecting patterning and the development of many organ systems (Wilson et al., 1953). The diversified biological functions of vitamin A are mediated by multiple levels of effectors including RAR, the retinoic acid receptor, and RXR, the retinoid X receptor (Chambon, 1996).

RAR and RXR are ligand-inducible transcription factors, regulating the transcription of an array of retinoid responsive genes through a bimodal mode (Lonard and O'Malley B, 2007). As a heterodimer, RAR and RXR bind constitutively to retinoic acid response elements (RARE) located within the regulatory region of retinoid responsive genes regardless of ligand (Chambon, 2005). In the absence of ligand, the DNA bound RAR and RXR heterodimer acts as a repressor of transcription by associating with the N-CoR corepressor complex, but acts as an activator by recruiting SRC and p300 coactivator complexes upon ligand induction. As a result, N-CoR is present at the RARE in the absence of ligand, whereas SRC and p300 are detected at RARE-regulated promoters following ligand induction (Folkers et al., 1998; Pavri et al., 2005). Thus, some retinoid responsive promoters are classified as pre-set or poised promoters, since Pol II and TBP bind to the TATA box constitutively (Pavri et al., 2005).

The transcriptional coactivator p300, initially identified as an E1A-associated protein contains an intrinsic histone acetyltransferase (HAT) activity and multiple

interaction surfaces for association with many transcription factors, activators and components of basal transcription machinery (Arany et al., 1995; Ogryzko et al., 1996). The function of p300 is critical for a broad array of biological processes including development, growth and cellular differentiation (Eckner et al., 1994; Torchia et al., 1998). Embryonic development is very sensitive to p300 gene dosage, and cells derived from p300 knockout embryos are defective in retinoid signaling (Yao et al., 1998). In addition, p300 also functions as a tumor suppressor and mutations in the p300 gene have been detected in many epithelial cancers (Gayther et al., 2000; Muraoka et al., 1996; Suganuma et al., 2002).

The 26S proteasome pathway is one of the major proteolysis systems of the cell. It contains a 20S core particle capped at both ends by the 19S regulatory particles which recognize and deliver ubiquitinated proteins to the 20S proteasome (Lee and Goldberg, 1998b). Many transcriptional activators, nuclear receptors and coactivators are subject to modification by ubiquitination or degradation through the proteasome pathway (Gianni et al., 2002; Kopf et al., 2000; Lange et al., 2000; Lonard et al., 2000; Molinari et al., 1999; Nawaz et al., 1999). Previously, we reported that histone deacetylase inhibitor sodium butyrate enhances p300 degradation through the 26S proteasome, which may account for some of the negative effects of butyrate on glucocorticoid-induced transcriptional activation (Li et al., 2002). We also reported that the histone deacetylase inhibitor-induced p300 degradation is mediated through the augmentation of gene expression of the B56γ3 regulatory subunit of protein phosphatase 2A, shedding new light on the molecular basis for the negative effects of histone deacetylase inhibitors on p300 function

(Chen et al., 2005). In addition, p300 is also a substrate of the cytoplasmic ubiquitin-proteasome system (Chen et al., 2007a).

The ubiquitin system plays a central role in diverse cellular processes including protein homeostasis, DNA repair and immune function (Ciechanover, 1998). Dysfunction of this system leads to various pathological conditions such as cancer, neurodegenerative diseases and immunological disorders (Schwartz and Ciechanover, 2009). In yeast, inhibition of the proteasome activity represses the expression of about 5% of the total active genes (Dembla-Rajpal et al., 2004). The effects of the 26S proteasome on gene transcription are mediated through either turnover of transcription factors or facilitation of transcription elongation (Lonard et al., 2000; Salghetti et al., 2001). It is known that the 26S proteasome activity is important for RAR-mediated transcriptional activation (Lonard et al., 2000). In addition, microinjection of an antibody against the 19S proteasome or pretreatment of cells with the proteasome inhibitor MG132 blocks ligand-induced transcriptional activation of RAR-β gene (Perissi et al., 2004). However, the precise role of the 26S proteasome in RAR-mediated transactivation remains unclear.

In this study, we determined that the proteasome activity is essential for protein-protein interaction of RAR with its coregulators such as SRC, p300 and RXR, for the promoter occupancy of liganded RAR, and consequently for the recruitment of the coactivator complex to the retinoid responsive promoters. In addition, the requirement of proteasome activity for the binding of liganded RAR to RARE is determined by the promoter context.

Materials and Methods

Cell culture and Reagents

Mouse embryonic carcinoma P19 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum and non-essential amino acids at 37ºC with 5% of CO2. TTNPB and MG132 were purchased from Sigma-Aldrich. PS-341 and cycloheximide were from LC Laboratory. Antibodies against RARα, RARβ, RXRα, N-CoR, SRC-1, p300, Pol II and ubiquitin were purchased from Santa Cruz Biotechnology. Protein A agarose/salmon sperm DNA was from Upstate.

Cell transfection and Luciferase assay

Transient transfections were performed with reporter plasmid by using ExGen 500 (Li et al., 2002). Luciferase assay was performed as previously described (Chen et al., 2004a). The luciferase activities are expressed as fold induction relative to the untreated controls after being normalized to the β-galactosidase activity.

Quantitative real-time RT-PCR

Total RNA was isolated by using RNeasy Mini Kit (Qiagen) and reverse transcribed by using High Capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time RT-PCR analysis was performed as previously described (Chen et al., 2006) by using the 7500 Fast Real-Time PCR System (Applied Biosystems). Gene specific primers and TaqMan probes used for the amplification were obtained from

Applied Biosystems. Quantification of mRNA levels was performed by using 18S rRNA as an internal control.

Whole cell extracts and Immunoprecipitation

Whole cell extracts were prepared by incubating the cells in whole cell extract buffer (10% glycerol, 50 mM Tris-HCl pH 7.6, 400 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF and 1% NP-40) for 30 minutes at 4˚C and then centrifuged at 14,000 rpm for 15 minutes at 4˚C. Bradford assay (Bio-Rad) was used to determine the protein concentrations. For immunoprecipitation assay, equal amounts of protein extracts were diluted with dilution buffer (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF and 1 mg/ml BSA) and incubated with antibodies specific to protein of interest for overnight at 4˚C. Protein A agarose were added to the incubation for additional 2 hours and the precipitates were then washed 3 times by PBS supplemented with 1% of Triton X-100.

Protein stability assays

Cells were labeled for 4 hours with 10 μCi/ml of ^{35}S-methionine/cysteine (PerkinElmer) in methionine-free media and the cells were chased for additional 4-24 hours in regular medium in the presence or absence of ligand. The cells were then harvested for the preparation of whole cell extracts and immunoprecipitation with a RARα antibody. The immunopurified RARα was separated by SDS PAGE. The gel was treated with amplify as manufacture recommended (Amersham). The apparent half-life was determined by the remaining incorporated radioactivity of the

immunoprecipitated RARα following various hours of chase. Quantification was performed by using a PhosphoImager (Molecular Dynamics). Alternatively, cells were treated with 10 μg/ml of cycloheximide in the presence or absence of ligand for 4-24 hours and harvested for quantitative Western analysis to determine the apparent half-life of RXRα.

Chromatin Immunoprecipitation assay

Cells were crosslinked with 1% formaldehyde for 15 minutes at 37˚C, lysed with lyses buffer (1% SDS, 50 mM Tris-HCl pH 8.0, 10 mM EDTA and 0.1% protease inhibitor cocktail) and sonicated with Bioruptor (Diagenode). Equal amount of DNA were diluted with dilution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 0.1% protease inhibitor cocktail) for immunoprecipitation with specific antibodies for overnight at 4˚C. Protein A agarose/salmon sperm DNA were then added to the incubation for additional 2 hours. The immune complexes were washed sequentially for 10 minutes with washing buffers A (0.1% SDS, 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA and 1% Triton X-100), buffer B (0.1% SDS, 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM EDTA and 1% Triton X-100), buffer C (1% sodium deoxycholate, 20 mM Tris-HCl pH 8, 0.25 M LiCl, 1 mM EDTA and 1% NP-40) and 2 times with TE buffer (10 mM Tris-HCl pH 8.0, and 1 mM EDTA). The immunocomplexes were then extracted with elution buffer (1% SDS and 0.1 M $NaHCO_3$) for 15 minutes at room temperature. Reverse cross linking were performed at 65˚C for overnight. The precipitated DNA fragments were then purified using QIAquick PCR

purification Kit (Qiagen) and amplified by PCR using the RAR-β2 and Cyp26A1 primers (Gillespie and Gudas, 2007b; Lefebvre et al., 2002a).

Results

The 26S proteasome activity is important for RAR mediated transcriptional activation

It is known that retinoic acid (RA) induced transcriptional activation requires the 26S proteasome activity (Perissi et al., 2004). However, it is not clear whether the proteasome activity is mediated solely through the action of ligand activated RAR or also through RXR, since all-trans RA used in previous studies can be metabolized into 9-cis RA and thus has affinity to both RAR and RXR. To decipher the role of the 26S proteasome in the function of RAR *per se* with respect to RARE-dependent gene activation, we employed TTNPB (4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid), a potent retinoic acid analog selective for all RAR subtypes (Bissonnette et al., 1995). We also used mouse pluripotent embryonal carcinoma P19 cells in which the transcription of RAR-β gene is rapidly induced by RA and the recruitment of p300 coactivator complex to the RARE region of the promoter is mediated by liganded RARα with RXRα acting as a silence partner (McBurney et al., 1982; Pavri et al., 2005).

We first used a luciferase reporter containing the RARE segment of RARβ2 promoter to examine the role of the 26S proteasome activity in TTNPB induced transactivation. The P19 cells were transfected with the RARβ2 reporter and induced with the RAR selective ligand in the presence or absence of MG132, a reversible proteasome inhibitor (Lee and Goldberg, 1998b), and then harvested for the luciferase assays. Consistent with previous reports, the RAR selective ligand induced

transcriptional activation of the reporter, by about 50 fold, while this transactivation was significantly inhibited, about 85%, by the addition of proteasome inhibitor MG132 (Fig. 1A).

We next examined the role of the 26S proteasome in the expression of endogenous RARβ gene, since proteasome inhibitors can reduce luciferase activity in tissue culture cells (Deroo et al., 2002). Real-time RT-PCR analysis revealed that treatment of the P19 cells with the RAR selective ligand for 16 hours increased the transcript level of RAR-β gene by about 30 fold, whereas inhibition of the 26S proteasome activity with MG132 reduced the accumulation of RARβ transcripts by about 60% (Fig. 1B). Thus the 26S proteasome participates in RARE-dependent gene expression through the regulation of RAR as an activator.

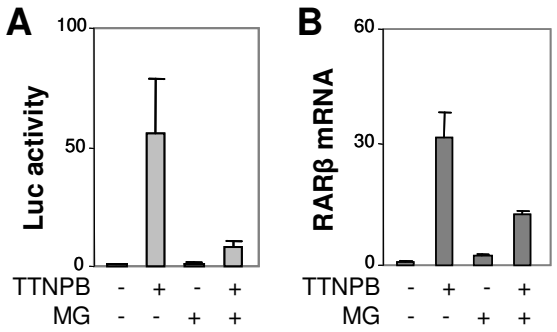

Figure 1. **RARE-dependent transcriptional activation depends on the 26S proteasome activity**

(A) The cells were transfected with a RARE reporter and then treated with TTNPB (1 μM) in the presence or absence of MG132 (MG, 5 μM) for 16 hours. Shown are fold inductions of the luciferase activities in relation to the untreated control. β-galactosidase activity was used as an internal control. Error bars represent the standard deviations of three independent experiments. (B) Real-time RT-PCR analysis of the levels of endogenous RARβ mRNA following 16 hours of ligand induction in the presence or absence of MG132, which are presented as fold variations compared to the untreated control. 18S rRNA was used as an internal control. Error bars represent the standard deviations of triplicates of one representative experiment.

Short term RAR selective ligand induction does not affect RAR stability

To determine if the RAR selective ligand targets RAR degradation through the 26S proteasome pathway, we also examined the impact of the RAR selective ligand on protein turnover of RARα and its co-regulators. The cells were induced with ligand in the presence or absence of MG132 for 4, 8 or 16 hours and subjected to quantitative Western analysis. As shown in figure 2A and 2B, the steady-state levels of RARα protein remained constant following treatment with the RAR selective ligand regardless proteasome inhibition for up to 16 hours. Similarly, the steady-state levels of RXRα, SRC-1 and p300 were also not decreased by these treatments (Fig. 2A and 2B). In contrast, the levels of N-CoR protein increased significantly by about 2 fold, compared to the untreated control cells following 4 or 8 hours of proteasome inhibition (Fig. 2A and 2B).

We next determined that the stability of RARα protein in response to the treatment with RAR selective ligand and proteasome inhibitor. In agreement with the literature, RARα is a fairly stable protein with an apparent half-life about 23 hours in the absence of selective ligand as determined by a pulse-chase protocol (Fig. 2C). Interestingly, the apparent half-life of RARα was not significantly affected by ligand induction *per se*, about 25 hours (Fig. 2C). RXRα is a more stable protein, so we used a cycloheximide protocol instead to determine the apparent half-life of RXRα. The cells were treated with cycloheximide which inhibits ribosome translocation (Obrig et al., 1971), for 4 to 24 hours and then harvested for quantitative Western analysis of RXRα. As shown in figure 2D, the apparent half life of RXRα is about 40 hours in the absence of RAR selective ligand. Again, the apparent half life of RXR was not significantly affected by ligand

induction, about 37 hours (Fig. 2D). Taken together, these data suggest that the 26S proteasome activity may participate in the activation of retinoid-responsive genes through the regulation of RAR as an activator rather than through the mechanisms of RAR degradation or protein turnover.

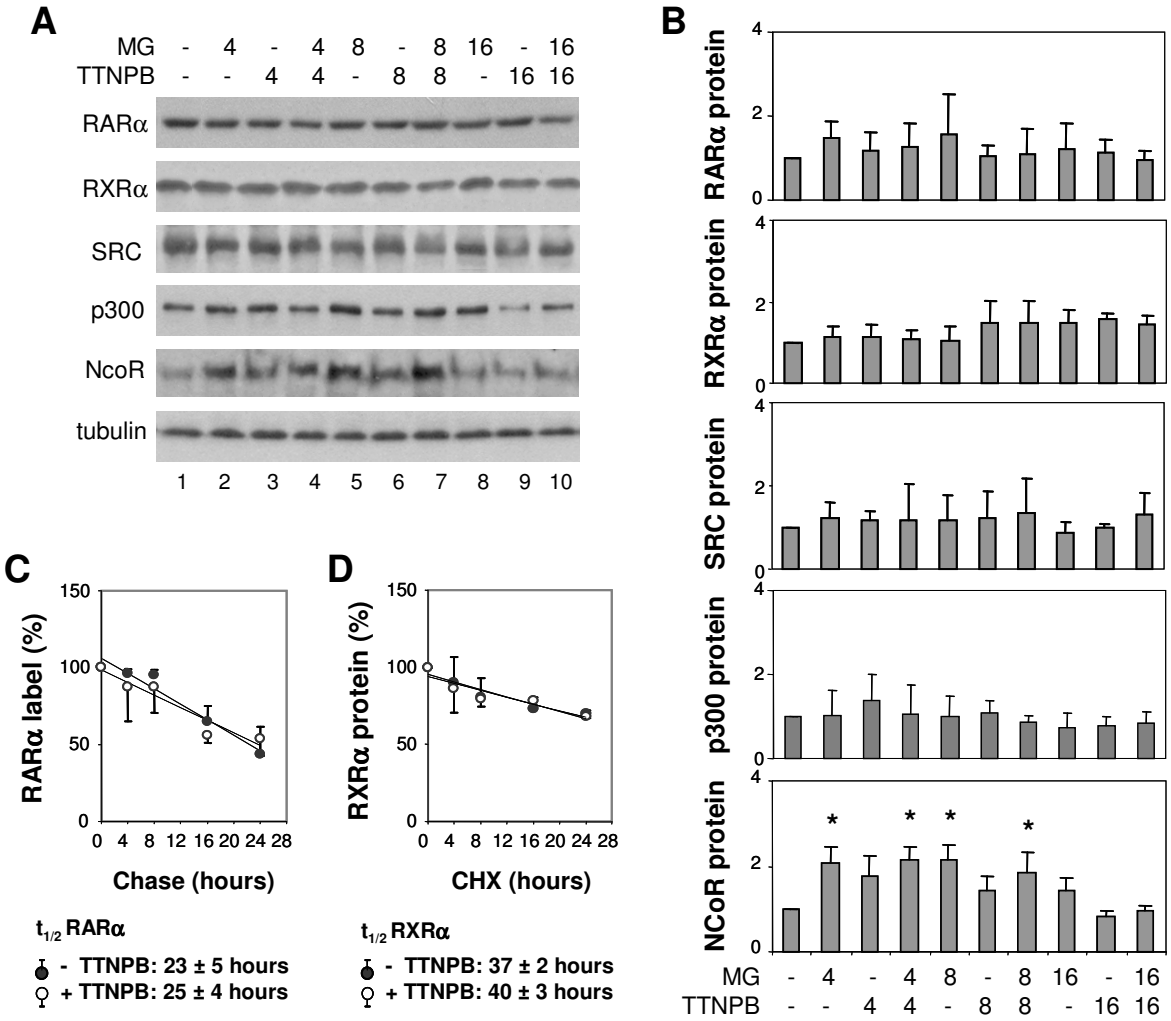
A
MG - 4 - 4 8 - 8 16 - 16
TTNPB - - 4 4 - 8 8 - 16 16
RARα
RXRα
SRC
p300
NcoR
tubulin
1 2 3 4 5 6 7 8 9 10
B
RARα protein
RXRα protein
SRC protein
p300 protein
NCoR protein
MG - 4 - 4 8 - 8 16 - 16
TTNPB - - 4 4 - 8 8 - 16 16
C
RARα label (%)
Chase (hours)
t1/2 RARα
- TTNPB: 23 ± 5 hours
+ TTNPB: 25 ± 4 hours
D
RXRα protein (%)
CHX (hours)
t1/2 RXRα
- TTNPB: 37 ± 2 hours
+ TTNPB: 40 ± 3 hours

Figure 2. **Short term of ligand induction does not affect the stability of RARα**

(A) Equal amounts of whole cell extracts (50 μg) were used for Western blot analysis of the endogenous RARα, RXRα, SRC-1, p300 and N-CoR proteins following treatments with TTNPB (1 μM) for 4, 8 and 16 hours in the presence or absence of MG132 (MG, 5 μM). The blots were then stripped and reprobed with a γ-tubulin antibody for internal control. (B) Quantitative analysis of the blots as in panel A is expressed as fold variations compared with the untreated controls after being normalized to the loading controls. Error bars represent standard deviations of at least three independent experiments (*$p < 0.05$). (C) After overnight pulse labeling, the cells were chased in the presence or absence of ligand for 4-24 hours. The endogenous RARα proteins were immunopurified, separated by SDS-PAGE and quantified by PhosphorImager. The apparent half-life of endogenous RARα is 23 $\pm$ 5 hours in the absence of ligand and 25 $\pm$ 4 hours in the presence of ligand (n=3). (D) The cells were treated with cycloheximide (10 μg/ml) in the presence or absence of ligand for 4-24 hours and harvested for Western analysis of the endogenous RXRα. The apparent half-life of endogenous RXRα is 37 $\pm$ 2 hours in the absence of ligand and 40 $\pm$ 3 hours in the presence of ligand (n=3).

RAR selective ligand enhances RAR ubiquitination

To define the role of the 26S proteasome in the posttranslational modification of RAR, we examined the ubiquitination of RARα and RXRα upon ligand induction with an *in vivo* ubiquitination assay (St-Germain et al., 2008), since RARα and RXRα are known substrates of ubiquitin conjugation and degraded through the 26S proteasome pathway (Tanaka et al., 2001b). The cells were first treated with MG132 for 4 hours in the absence or presence of RAR selective ligand. The endogenous RARα or RXRα protein was immunopurified with antibodies specifically against RARα or RXRα and then subjected to Western analysis. The RARα or RXRα blots were first probed with an ubiquitin antibody to examine the ubiquitination status of these proteins, and reprobed with specific antibodies against RARα and RXRα for internal controls.

As shown in figure 3A and 3B, the ubiquitin signal of the RARα immunoprecipitates became readily detectable following 4 hours of proteasome inhibition in the absence of ligand, about 5 fold in comparison to the untreated control. Following the addition of the RAR selective ligand, the level of RARα ubiquitination increased significantly, more than 3 fold when compared with the MG132 only treatment (Fig. 3A and 3B). The level of RXRα ubiquitination was also readily detectable following 4 hours of proteasome inhibition, about 6 fold in relation to the untreated control (Fig. 3C and 3D). However, the degree of RXRα ubiquitination was not significantly affected by the addition of RAR selective ligand (Fig. 3C and 3D). Collectively, these data suggest that the RAR selective ligand play a specific role in RAR ubiquitination and the 26S proteasome regulates the activator activity of RAR through an ubiquitin-dependent pathway.

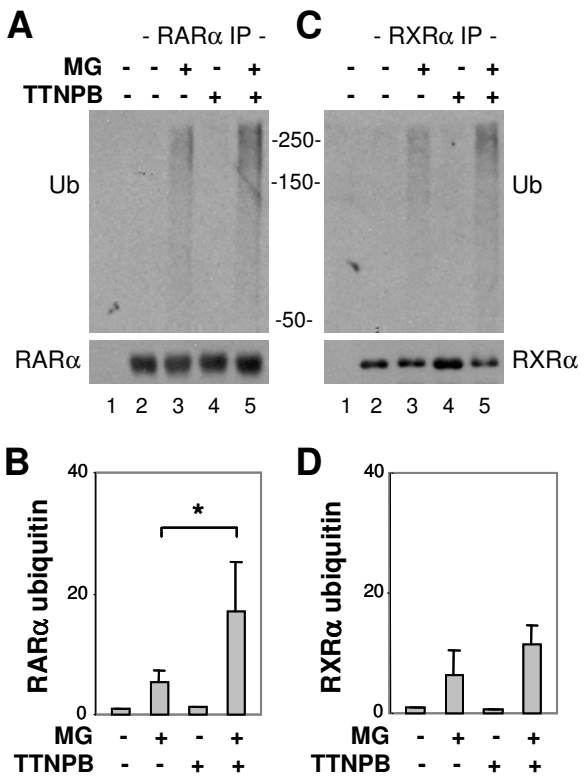

Figure 3. **RAR selective ligand enhances RARα ubiquitination**

(A) An *in vivo* ubiquitin assay was used to assess the levels of RARα ubiquitin conjugation following 4 hours of treatment with MG132 (MG, 5 μM) in the presence or the absence of TTNPB (1 μM). The blots were first probed with an ubiquitin-specific antibody, and then stripped and reprobed for RARα as internal controls. (B) Quantification of the Western blots as in panel A is presented as average fold variation of the ubiquitin signal normalized to the levels of immunopurified RARα following ligand induction and MG132 treatments in comparison to the MG132 only control. Error bars are the standard deviations of three independent experimental repeats (*$p < 0.05$). (C) and (D) The experimental procedures were as panel (A) and (B) except that the ubiquitination of RXRα protein was studied.

The 26S proteasome activity is important for ligand-dependent interaction of RAR with its coregulators

To determine the impact of proteasome on ligand induced RAR conformation changes, we examined the association of RAR with its coactivators since one important aspect of RAR acting as activator is to recruit coactivators to the RARE regions of genomic DNA through protein-protein interaction. First, the cells were treated with RAR selective ligand in the presence or absence of MG132 for 4 hours and the endogenous SRC-1 were immunoprecipitated with a specific antibody against SRC-1 and subjected to Western analysis. The blots were first probed with an antibody against RARα to detect the endogenous RARα coimmunoprecipitated with SRC-1, and then striped and reprobed for RARα as an internal control. As shown in figure 4A, the association of RARα with SRC-1 depends on RAR selective ligand and this ligand-dependent interaction of RARα with SRC-1 was disrupted following 4 hours of MG132 administration compared with the ligand alone treatment.

We next examined the interaction of RARα with endogenous p300 by using a specific antibody against p300 with the same co-immunoprecipitation approach. As shown in figure 4B, the interaction of RARα with p300 also depends on ligand induction. Similarly, the ligand-dependent interaction of RARα with p300 was also disrupted following 4 hours of proteasome inhibition (Fig. 4B). In addition, the association of RARα with RXRα was also disrupted following 4 hours of MG132 addition compared to the TTNPB alone treatment (Fig. 4C). Thus, the activity of the 26S proteasome may be important for of RARα to interact with its coregulators during ligand-induced transcriptional activation.

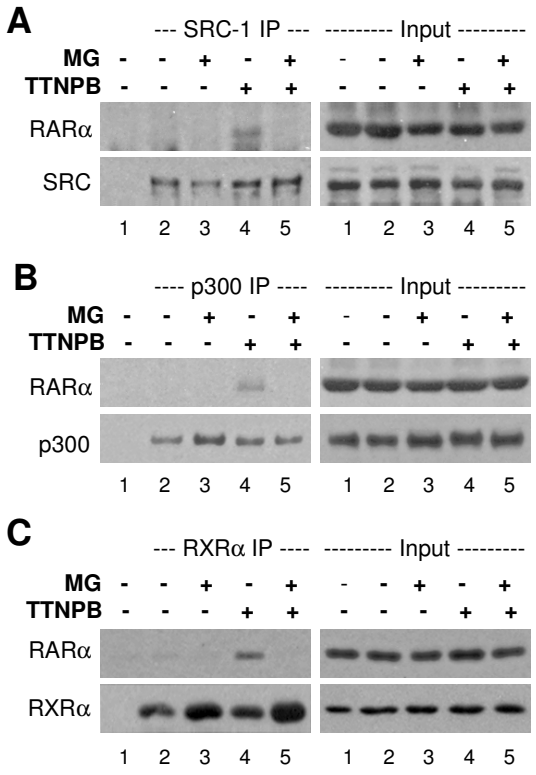

Figure 4. **The 26S proteasome activity is important for ligand-dependent interaction of RARα with its coactivators**

(A) Coimmunoprecipitation of endogenous RARα with SRC-1 was examined following 4 hours of TTNPB induction (1 μM) in the presence or absence of MG132 (MG, 5 μM). The Western blots were first probed with a RARα antibody, and then stripped and reprobed for SRC-1. Lane 1 is a negative immunoprecipitation control. 10% of the input extracts were also subject to Western analysis as internal controls. Shown are the representatives of three independent experiments. (B) Coimmunoprecipitation of endogenous RARα with p300 following the same treatments. Western blots were first probed with a RARα antibody and then reprobed for p300. (C) Coimmunoprecipitation of endogenous RARα with RXRα was also examined following the same treatment. The Western blots were first probed with a RARα antibody then reprobed for RXRα.

The 26S proteasome is critical for ligand-dependent occupancy of RARβ2 promoter

To delineate the role the 26S proteasome in the activator activity of RAR, we employed the RARβ2 promoter as a model system as it contains a classic DR5 RARE in the vicinity of TATA box (Fig. 5A). The cells were induced with ligand in the presence or absence of MG132 for 1, 2 and 4 hours since the association of RARα with its coactivators was disrupted following 4 hours of proteasome inhibition (Fig. 4). A time course real-time RT-PCR analysis revealed that RAR-β gene transcription was rapidly induced by the addition of RAR selective ligand up to 15 fold by 4 hours, whereas inhibition of the 26S proteasome activity impaired the increase of RARβ transcripts by about 50% (Fig. 5B). Most intriguingly, the decrease in RARβ mRNA levels was observed only after 2 hours of cotreatment with MG132 (Fig. 5B).

We next examined the occupancy of the RARβ2 promoter by N-CoR, because corepressor release is a prerequisite for transcriptional activation of the retinoid responsive genes (Kao et al., 2003). Consistent with previous reports, N-CoR was detected at the RARβ2 promoter in the absence of ligand and was immediately released from the RARE upon ligand induction as shown by the time course chromatin immunoprecipitation (ChIP) analysis (Fig. 5C). Interestingly, inhibition of proteasome activity did not disrupt the ligand-independent association of N-CoR to the promoter, rather increased this association to a comparable level with the augmentation of N-CoR protein following MG132 treatment (compare Fig. 5C-5D with Fig. 2A-2B).

We also examined the binding of RARα and RXRα to the RARβ2 promoter by using the same time course ChIP analysis. In line with the literature, the association of RARα and RXRα to the RARE was constitutive, regardless of ligand (Fig. 5C, 5E and

5F). Moreover, treatment of the cells with MG132 in the absence of ligand did not perturb the ligand-independent occupancy of RARE by RARα and RXRα (Fig. 5C, 5E and 5F). However, in the presence of RAR selective ligand, MG132 treatment impeded significantly the binding of RARα and RXRα to the RARE (Fig. 5C, 5E and 5F). Most intriguingly, this impediment only became significant after 2 hours of MG132 administration, which is concomitant with the decrease in RARβ gene transcripts (Fig. 5B, 5C, 5E and 5F). Following 4 hours of treatment, the RARE bound RARα and RXRα was reduced by about 90% (Fig. 5C, 5E and 5F).

To further assess the impact of the 26S proteasome on the coactivator occupancy of the RARβ2 promoter, we also performed the time course ChIP analysis with specific antibodies against SRC-1, p300 and Pol II. As shown in figure 5C, SRC-1 was detected at the RARE following 1 hour of ligand induction, whereas the association of p300 to the RARE was observed later, following 2 hours of induction. However, the ligand-dependent recruitment of SRC-1 and p300 was disrupted in a time-dependent manner when the cells were treated with proteasome inhibitor MG132 (Fig. 5C). Again, the loss of SRC-1 and p300 recruitment was seen after 4 hours of treatment, coinciding with the perturbation of RAR and RXR occupancy at the promoter and the decrease in RARβ gene transcripts (Fig. 5B, 5C, 5E and 5F). However, the association of Pol II at the promoter was not affected by the MG132 treatment, regardless of ligand (Fig. 5C).

To determine whether the 26S proteasome is required for the maintenance of the activator activity of RAR in general or specifically required for the process of ligand activation, we also designed a MG132 pretreatment protocol, as the association of liganded RAR to RARE promoter was impeded only after 2 hours of MG132 addition

(Fig. 5C). The cells were first treated with MG132 alone for 2 or 3 hours, and then together with RAR selective ligand for an additional hour. As shown in figure 5G, the loss of liganded RARα or the silent partner RXRα at the RARE is correlated with the hours of MG132 treatment rather than ligand induction. Taken together, these data suggest that the 26S proteasome activity is important for the binding of the receptor to the RARE in response to ligand induction possibly through modulation of the receptor conformation change or ubiquitination.

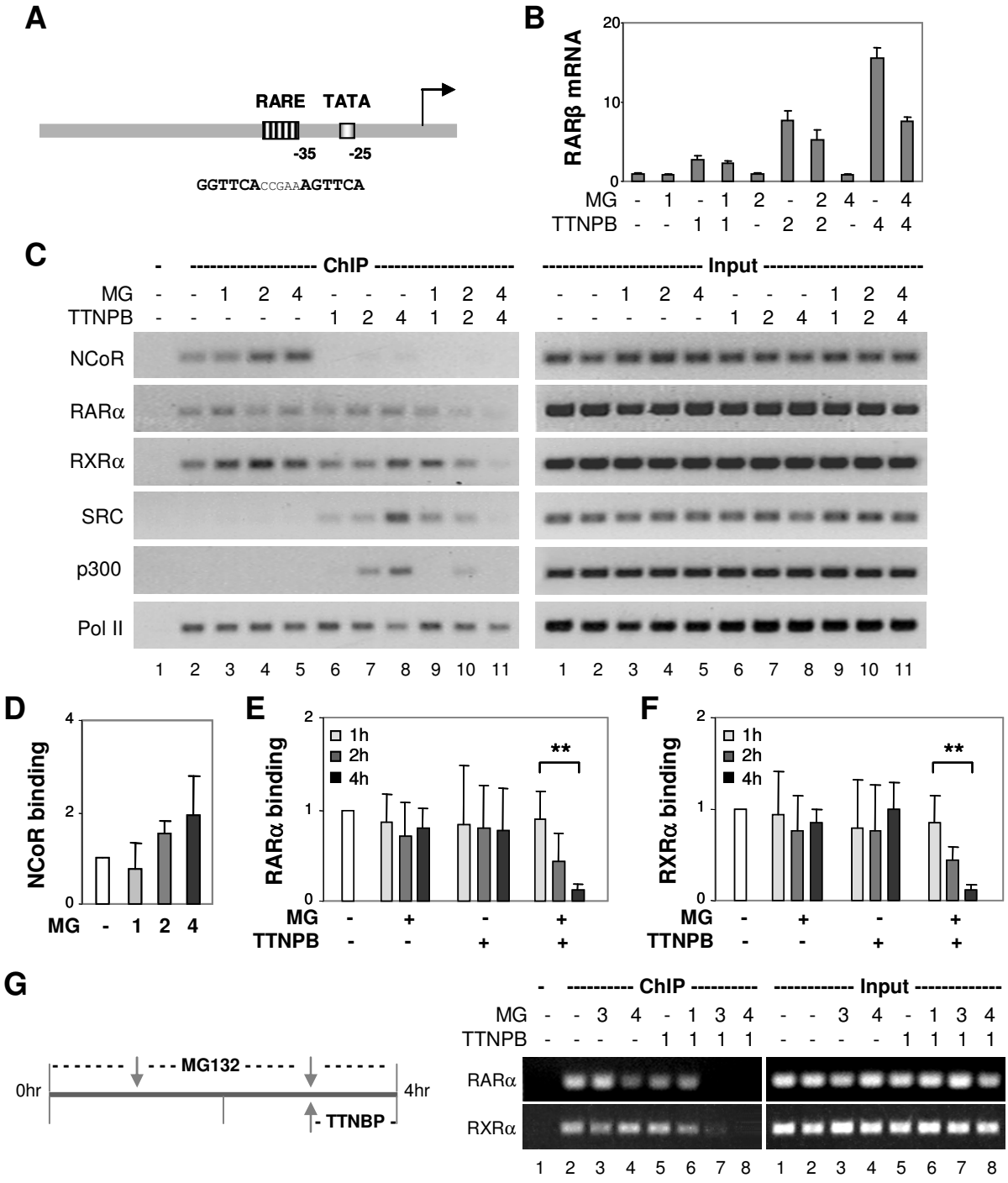
A
RARE TATA
-35 -25
GGTTCACCGAAAGTTCA
B
RARβ mRNA
MG - 1 - 1 2 - 2 4 - 4
TTNPB - - 1 1 - 2 2 - 4 4
C
ChIP
Input
MG - - 1 2 4 - - - 1 2 4
TTNPB - - - - - 1 2 4 1 2 4
NCoR
RARα
RXRα
SRC
p300
Pol II
1 2 3 4 5 6 7 8 9 10 11
D
NCoR binding
MG - 1 2 4
E
RARα binding
1h
2h
4h
**
MG - + - +
TTNPB - - + +
F
RXRα binding
G
MG132
0hr
4hr
- TTNBP -
RARα
RXRα
1 2 3 4 5 6 7 8

Figure 5. **The 26S proteasome activity is essential for ligand-dependent occupancy of RARβ2 promoter**

(A) Schematic presentation of the relation of the DR5 RARE and TATA box at the promoter. (B) Real-time RT-PCR analysis of the levels of RARβ mRNA in cells treated with TTNPB (1 μM) in the presence or absence of MG132 (MG, 5 μM) for 1, 2 and 4 hours. Quantification is presented as fold variations compared to the untreated control. 18S rRNA was used as an internal control. Error bars represent the standard deviations of triplicates from one representative experiment. (C) ChIP analysis of the occupancy of RARβ2 promoter by N-CoR, RARα, RXRα, SRC-1, p300 and Pol II following 1, 2 and 4 hours of ligand induction in the presence or absence of MG132. Lane 1 is the negative ChIP control. The input DNA was also analyzed in parallel. (D) - (F) Quantitative analysis of the association of N-CoR, RARα and RXRα to the RARE is expressed as fold variations compared with the untreated controls after being normalized to the input controls. Error bars represent standard deviations of five independent experiments (**$p < 0.01$). (G) Following pretreatments with MG132 for 2 and 3 hours and together with ligand for an additional hour, the occupancy of RARα and RXRα was examined by ChIP analysis.

The role of promoter context in liganded RAR occupancy

To define the determinants for the role of proteasome in the occupancy of liganded RAR at RARE, we employed another RA target gene, Cyp26A1 (Loudig et al., 2005). The Cyp26A1 gene contains two well defined DR5 RARE, one (R1) is close to the TATA box and another (R2) about 2 kb upstream (Fig. 6A). The cells were treated with ligand in the presence or absence of MG132 for 1 and 4 hours and subjected to real-time RT-PCR analysis. As shown in figure 6B, the Cyp26A1 transcription was robustly induced by RAR selective ligand, about 60 fold by 4 hours, whereas inhibition of the 26S proteasome activity impaired the accumulation of Cyp26A1 transcripts by about 70%. Most interestingly, the decrease in Cyp26A1 mRNA level was observed only at 4 hours of cotreatment with MG132 (Fig. 6B).

Consistent with previous reports, the occupancy of RARα and RXRα was detected at the R1 and R2 region of the Cyp26A1 promoter regardless of ligand as shown by the ChIP analysis (Fig. 6C). In the absence of RAR selective ligand, inhibition of proteasome activity did not disrupt the association of RARα and RXRα to the R1 or R2 region (Fig. 6C). However, in the presence of ligand, MG132 treatment impeded significantly the binding of RARα and RXRα to the R1 region but not the R2 region (Fig. 6C). Again, this impediment of R1 occupancy only became apparent after 4 hours of MG132 administration, concomitant with the decrease in Cyp26A1 gene transcripts (Fig. 6B-6C). Taken together, these data suggest that the proximity of RARE to the TATA box, or the looping of distal RARE to the proximal promoter may be a factor for the involvement of proteasome in the binding of liganded receptor to the RARE.

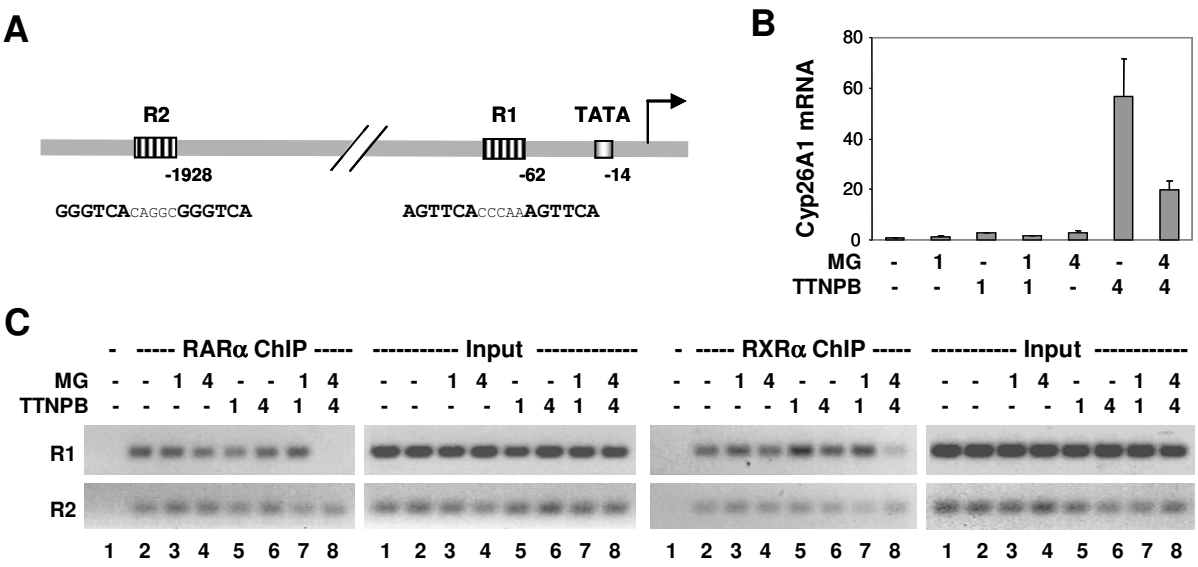

Figure 6. **The 26S proteasome activity is essential for ligand-dependent occupancy of R1, but not R2 region of Cyp26A1 promoter**

(A) Schematic presentation of the R1 and R2 regions relative to the TATA box at Cyp26A1 promoter. (B) Real-time RT-PCR analysis of the levels of Cyp26A1 mRNA in cells treated with TTNPB (1 μM) in the presence or absence of MG132 (MG, 5 μM) for 1 and 4 hours. Quantification is presented as fold variations compared to the untreated control. Error bars represent the standard deviations of the triplicates from one representative experiment. (C) ChIP analysis of the occupancy of Cyp26A1 regulatory region by RARα and RXRα following 1 and 4 hours of ligand induction in the presence or absence of MG132. Lane 1 is the negative ChIP control. The input DNA was also analyzed in parallel.

Proteasome inhibitor PS-341 has the same effects as MG132 on the occupancy of RARE by liganded RAR

To determine the specificity of proteasome activity in the occupancy of liganded RAR at RARE, we also employed another potent proteasome inhibitor, PS-341 which is well tolerated in clinical trials (Adams, 2002). Western analysis demonstrated that treatment with 10 or 20 nM of PS341 for up to 8 hours did not affect the steady-state levels of RARα and RXRα (Fig. 7A). We then examined the effects of PS341 on RARE dependent gene expression. The cells were induced with ligand in the presence or absence of PS-341 for 1 and 4 hours as the occupancy of RARα and RXRα at the RARβ2 or R1 region of Cyp26A1 was disrupted following 4 hours of proteasome inhibition (Fig. 5 and Fig. 6). PS-341 reduced the level of RARβ mRNA, about 50% with a similar degree as MG132, and this reduction only became evident after 4 hours of PS-341 administration (compare Fig. 7B with Fig. 5B). In addition, the effect of PS-341 on the transcript level of Cyp26A1 gene was also similar to MG132 (compare Fig. 7B with Fig. 6B). Interestingly, the transcripts of Cyp26A1 gene appear to be more sensitive to the MG132 and PS-341 than that of RARβ (Fig. 5B, 6B and 7B).

We next examined the effects of PS-341 on RARE occupancy. Similar to MG132, PS-341 did not affect the binding of unliganded RARα to the RARβ2 or the R1 region of Cyp26A1, but disrupted the RARE occupancy of RARα in the presence of the RAR selective ligand, (Fig. 7C). Again, this impediment only became apparent after 4 hours of PS-341 administration coinciding with the decrease in gene specific transcripts (Fig. 7B-7C). In addition, the effects of PS341 on the binding of liganded RARα to the R2 region of Cyp26A1 and on the occupancy of RXRα at these RARE regions were also similar to

MG132 (Fig. 7C-7D). Collectively, these data demonstrated that the proteasome activity is essential for the binding of liganded RARα to RARE in the vicinity of TATA box.

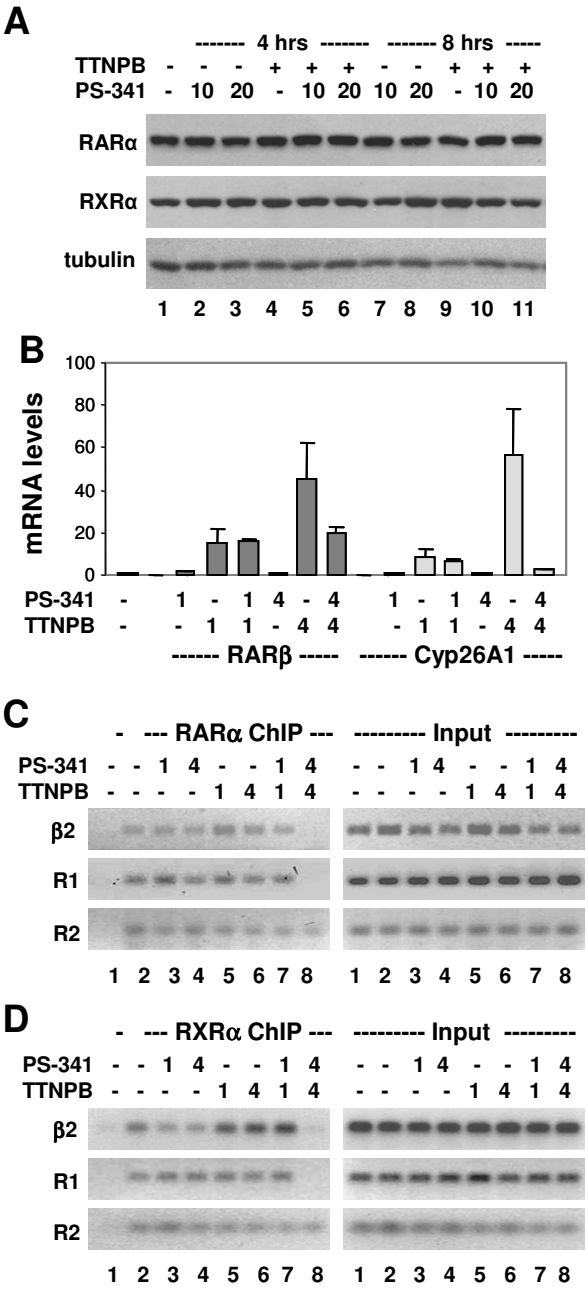
A
------- 4 hrs ------- ------- 8 hrs -----
TTNPB - - - + + + - - - + + +
PS-341 - 10 20 - 10 20 10 20 - 10 20
RARα
RXRα
tubulin
1 2 3 4 5 6 7 8 9 10 11
B
mRNA levels
100
80
60
40
20
0
PS-341 - 1 - 1 4 - 4 1 - 1 4 - 4
TTNPB - - 1 1 - 4 4 - 1 1 - 4 4
------ RARβ ----- ------ Cyp26A1 -----
C
- --- RARα ChIP --- --------- Input ---------
PS-341 - - 1 4 - - 1 4 - - 1 4 - - 1 4
TTNPB - - - - 1 4 1 4 - - - - 1 4 1 4
β2
R1
R2
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
D
- --- RXRα ChIP --- --------- Input ---------
PS-341 - - 1 4 - - 1 4 - - 1 4 - - 1 4
TTNPB - - - - 1 4 1 4 - - - - 1 4 1 4
β2
R1
R2
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8

Figure 7. **Effects of proteasome inhibitor PS-341 on RAR-mediated transcriptional activation**

(A) Cells were treated with TTNPB (1 μM) in the presence or absence of PS-341 (PS, 10 or 20 nM)) for 4 or 8 hours and then subjected to Western analysis of RARα and RXRα protein. The blots were then stripped and reprobed for γ-tubulin as an internal control. (B) Real-time RT-PCR analysis of the levels of RARβ and Cyp26A1 mRNA in cells treated with TTNPB and PS-341 (PS, 10 nM)) for 1 and 4 hours. Quantification is presented as fold variations compared to the untreated control. Error bars represent the standard deviations of the triplicates from one representative experiment. (C) ChIP analysis of the occupancy of RARβ2 and R1 and R2 region of Cyp26A1 by RARα in cells treated with TTNPB and PS-341. Lane 1 is the negative ChIP control. The input DNA was also analyzed in parallel. (D) The binding of RXRα was also analyzed by the ChIP assay.

Discussion

In this study, we examined the impact of the 26S proteasome on the expression of retinoid responsive genes and determined the molecular basis for the role of the proteasome in retinoid-induced transcriptional activation. First, the 26S proteasome plays an important role in the regulation of RARα to serve as an activator. Second, the proteasome activity is essential for ligand-dependent interaction of RAR with its co-regulators such as SRC-1, p300 and RXR. Third, the proteasome activity is required for the occupancy of RARE by RARα and RXRα upon ligand induction, consequently for the recruitment of coactivator complex in the vicinity of TATA box. Thus, the promoter context determines the involvement of proteasome in regulation of RARα as an activator.

The importance of the 26S proteasome in the activation of hormone responsive genes was initially established for estrogen-induced gene activation (Lonard et al., 2000). Subsequent studies have revealed the ubiquitination and degradation of several nuclear receptors as well coactivators during the course of their nuclear activities (Nawaz and O'Malley, 2004). Proteins related to the 26S proteasome pathway have also been found at the promoter regions of hormone responsive genes (Perissi et al., 2004). Collectively, these studies appear to suggest that protein degradation is coupled with the activator activity of nuclear receptors and that the ubiquitin-proteasome pathway modulate transcription by promoting turnover of the nuclear receptor-transcription complex. However, it is not clear at which molecular level the 26S proteasome regulates the activator activities of nuclear receptors and whether the proteasome is required for the activator activity or just the receptor turnover.

In this study, we focused on the molecular basis for the involvement of the 26 proteasome pathway in RARα-mediated transcriptional activation by using a RAR selective ligand and two well established promoter systems in which ligand rapidly induces the expression of RARβ and Cyp26A1 genes. Our observations that ligand does not promptly enhance RAR turnover, and that the 26S proteasome activity is required for the binding of liganded RAR to the genomic DNA RARE (Fig. 2-7), are suggestive of a non-proteolytic role of proteasome in RARα-mediated gene activation. Such a role for proteasome has emerged recently (Chen and Sun, 2009).

Ligand induced receptor degradation has been reported for many nuclear receptors such as estrogen receptor, progesterone receptor, thyroid hormone receptors, RAR and RXR (Lange et al., 2000; Nawaz et al., 1999; Tanaka et al., 2001b). The turnover of these receptors appears to all having some links with the proteasome pathway. In the case of RAR or RXR, the ligand induced receptor degradation is often observed after extended ligand treatments (Gianni et al., 2002; Kopf et al., 2000; Tanaka et al., 2001b). We did not detect negative effects of ligand on RARα protein following short period of treatment, 4-16 hours (Fig. 2) and RARα-mediated transactivation was significantly affected within 4 hours of proteasome inhibition (Fig. 5-7). Hence, our data indicate a role for the proteasome in the involvement of RARα to function as an activator through a proteolysis-independent pathway. However, ligand promptly enhances the ubiquitination of RAR (Fig. 3), suggesting that the 26S proteasome may be also involved in the function of RAR through proteolysis-dependent manner. However, what type of ubiquitination is engaged, where the ubiquitination occurs, or which E3 ligase is involved in this process remains to be determined.

Besides nuclear receptors, the destruction of other activators have also been intimately linked to transcriptional potential of these proteins (Molinari et al., 1999; Salghetti et al., 2001; Salghetti et al., 2000). While proteasome may play a dual role in transcriptional regulation through a proteolysis-dependent pathway, studies using yeast as model system have demonstrated that the proteasome pathway can also participate in the regulation of gene expression in a proteolysis-independent manner (Ferdous et al., 2007; Lee et al., 2005). Thus, it is possible that the 26S proteasome system targets the ubiquitinated receptors for degradation subsequent to transcriptional activation.

Transcriptional activation mediated by RAR depends on the cooperation of RXR to form a heterodimer at the RARE to recruit coactivator complex (Perlmann et al., 1993). Although, unliganded RAR is able to form a heterodimer with RXR as the core of corepressor complex at the RARE, the interaction of RAR with RXR in the absence of DNA is dependent on ligand induction (Depoix et al., 2001). In addition, the dimerization interface of RAR with RXR determines the cooperative binding of the heterodimer to DNA (Zechel et al., 1994). It has been proposed that ligand controlled dimerization is important for RAR activation process and depends on remodeling of the ligand domain of RAR (Depoix et al., 2001). Our study shows that the proteasome pathway is involved in RARα activation process through modulating the interaction of RARα with its coregulators, and consequently the formation of heterodimer on the genomic DNA (Fig. 4 -7). Further mechanistic studies would be essential for elucidating the mechanisms underlying the complex dynamics of RAR genomic association, transcriptional activation and subsequent turnover.

In conclusion, our study shields new lights on how the activator activity of RARα is regulated during ligand-induced transcriptional activation and provides molecular basis for the involvement of the 26 proteasome pathway in this process.

Abbreviations

ChIP: Chromatin immunoprecipitation

HAT: histone acetyltransferase

HDAC: histone deacetylase

RAR: Retinoic acid receptor

RARE: Retinoic acid response element

RXR: Retinoid X receptor

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