14-3-3 sigma interacts with liver X receptor beta

Emily Ann Jackson

Louisiana State University and Agricultural and Mechanical College

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A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Biological Sciences

by

Emily Ann Jackson
B.S., Louisiana State University, 2004
May 2010
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LIST OF ABBREVIATIONS

NR- nuclear receptor
DBD- DNA binding domain
LBD- ligand binding domain
LXR- liver x receptor
NR1H2-nuclear receptor subfamily 1, group H, member 2 (LXRβ)
NR1H3-nuclear receptor subfamily 1, group H, member 3 (LXRα)
ABCA1- ATP binding cassette subfamily A member 1
SREBP-1C- sterol regulatory element binding protein 1C
FAS- fatty acid synthase
AR- androgen receptor
ER- estrogen receptor
GR- glucocorticoid receptor
MR- mineralcorticoid receptor
PR- progesterone receptor
FXR- farnesoid X receptor
PPAR- peroxisome proliferator-activated receptor
RAR- retinoid receptor
TR- thyroid hormone receptor
VDR- vitamin D receptor
Rev-erb- reverse orientation c-erb
ROR- retinoic acid receptor-related orphan receptor
FOXO- forkhead box
LDL- low density lipoproteins
HDL- high density lipoproteins
FDA- U.S. Food and Drug Administration
HNF-4- hepatocyte nuclear factor 4
SF-1- steroidogenic factor
SHP- small heterodimeric partner
RIP140- receptor interacting protein 140
NCoR- nuclear receptor corepressor
SMRT- silencing mediator of retinoic acid and thyroid hormone receptor
PBS- phosphate buffer saline
RCT- reverse cholesterol transport
ABSTRACT

Atherosclerosis is the leading cause of mortality in developed countries accounting for 50% of all deaths. Atherosclerosis develops when macrophages in the artery wall accumulate large amounts of cholesterol via uptake of oxidized low density lipoproteins (LDL), causing a negative effect on cholesterol metabolism. Thus, the development of atherosclerosis can be inhibited by increasing cholesterol efflux, which can be achieved by activating ATP binding cassette (ABC) transporters in macrophages. In particular, ABCA1 mediates reverse cholesterol transport to the liver via high density lipoproteins (HDL) and therefore is an attractive molecular target for raising HDL levels and protecting against atherosclerosis. ABCA1 gene expression is known to be regulated by various transcription factors, such as liver X receptor (LXR). LXR are transcription factors that are activated via binding of ligands, which are oxysterols. Activated LXR bind to promoter regions at specific sequences known as LXR response elements (LXRE) and regulate genes for cholesterol metabolism and transport, as well as for lipogenesis. Synthetic LXR ligands might be useful for treatment of atherosclerosis if they did not induce lipogenesis, which can lead to an accumulation of cholesterol via activation of steroid response element binding protein (SREBP)-1c in the liver. To develop selective treatments for atherosclerosis, we need to understand mechanisms of selective gene regulation. LXR occur as two isotypes, LXRα (NR1H3) and LXRβ (NR1H2). Both isotypes regulate genes encoding proteins involved in cholesterol metabolism and transport, as well as in lipogenesis. However, knock-out studies have shown that LXRβ activation results in more effective gene activation in the periphery, such as in macrophages, which can promote cholesterol efflux. The mechanism of this LXR isotype-selectivity is poorly understood. In this document, we will show how protein-protein
interactions affect LXRα and LXRβ function and explore the mechanism of nuclear export of LXRα and LXRβ.
CHAPTER 1

GENERAL INTRODUCTION

1.1 Nuclear Receptors

1.1.1 Introduction

Nuclear receptors are a large superfamily of transcription factors that regulate genes important to physiological functions such as homeostasis, reproduction, and development in target cells (Table 1.1). Nuclear receptor activity can be modified by binding to ligands, typically small biologically active compounds such as hormones and lipids. A few well known nuclear receptor ligands are hormones such as steroids, retinoids, thyroid hormone, and vitamin D3. A large number of nuclear receptors have been identified by amino acid sequence similarity to known receptors, but some have no identified natural ligand and are referred to as nuclear orphan receptors. Nuclear receptors are found within the cytoplasm and nucleus of the cell where they can directly regulate target genes by binding to a specific sequence on DNA, known as the response element, usually found within the promoter region of the target gene. Each response element consists of two half-sites of a consensus sequence which can be oriented as direct repeats or indirect repeats which allows nuclear receptors to bind as a monomer, homodimer, or heterodimer (Giguere 1999). The expression of a large number of genes is regulated by nuclear receptors. Many of these regulated genes are associated with major diseases such as cancer, diabetes, obesity, and atherosclerosis, which explain why nuclear receptors are used as molecular targets of approximately 13% of U.S. Food and Drug Administration (FDA) approved drugs (Overington, Al-Lazikani et al. 2006; Simmons 2006). Some of the top prescription drugs on the market target a NR including the drugs bicalutamide (Casodex), an androgen receptor (AR) ligand used to treat conditions for prostate cancer;
Spironolactone (Aldactone), a mineralcorticoid receptor (MR) ligand for heart failure; levothyroxine (Synthroid), thyroid receptor (TR) ligand for hypothyroidism; bexarotene

**Table 1.1** Nuclear Receptor involvements in physiology

<table>
<thead>
<tr>
<th>Physiological functions</th>
<th>Implicated NRs</th>
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<tr>
<td>Reproduction</td>
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<td>Fetal Development</td>
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<td>Cardiovascular homeostasis</td>
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<td>Skeletal homeostasis</td>
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<td>Central nervous system homeostasis</td>
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<td>Immunomodulation</td>
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<tr>
<td>Hematopoiesis</td>
<td>ERβ and RAR</td>
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<tr>
<td>Inflammation</td>
<td>GR, RAR, PPAR, and LXR</td>
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<tr>
<td>Wound healing</td>
<td>PPARα and PPARδ</td>
</tr>
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<td>Glucose homeostasis</td>
<td>ER, GR, and PPAR</td>
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<tr>
<td>Lipid and cholesterol homeostasis</td>
<td>VDR, TR, PPAR, LXR, FXR, CAR, and PXR</td>
</tr>
<tr>
<td>Toxin detoxification</td>
<td>CAR and PXR</td>
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</table>

(Targretin), an retinoid X receptor (RXR) ligand for skin cancer; and rosiglitazone (Avandia), a peroxisome proliferator-activated receptor (PPAR) ligand used for diabetes. The search for ligands for orphan receptors has become an active research area because of the potential to be used as therapeutic targets. For example, reverse orientation c-erb (Reverbs) and Retinoic acid receptor-related orphan receptor (ROR) have been de-orphanized but are still considered to be nuclear receptor due to the discovery of their ligands (Raghuram, Stayrook et al. 2007; Meng, McMaster et al. 2008; Rogers, Ying et al. 2008; Kumar, Solt et al. 2010; Wang, Kumar et al. 2010).
1.1.2 Classification and Nomenclature of NRs

1.1.2.1 Classification

The nuclear receptors are found across a variety of species. There are 48 nuclear receptor genes that are found within the human genome, 21 within the *Drosophila melanogaster* genome, and 270 within the *Caenorhabitis elegans* genome (Laudet 1997; Giguere 1999; Maglich, Sluder et al. 2001; Robinson-Rechavi, Carpentier et al. 2001). These nuclear receptors are classified into four classes such as Type I Nuclear Receptors, Type II Nuclear Receptors, Type III Nuclear Receptors and Type IV Nuclear Receptors based on their ligand and DNA binding characteristics. The Type I NRs are the classic steroid hormone receptors which include the androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR), mineralcorticoid receptor (MR), and the progesterone receptor (PR) (Figure 1.1). Type II NRs are non-steroid receptors that heterodimerize with RXR which include farnesoid X receptor (FXR), liver x receptor (LXR), peroxisome proliferator-activated receptor (PPAR), retinoid receptor (RAR), thyroid hormone receptor (TR), and the vitamin D receptor (VDR). The Type III and Type IV NRs are the orphan nuclear receptors which lacked a known physiological ligand when they were identified and can either bind DNA as a monomer or dimer. Type III NRs are dimeric orphan receptors that include hepatocyte nuclear factor 4 (HNF-4), chicken ovalbumin upstream promoter (COUP), and germ cell nuclear factor (GCNF). The Type IV NRs are monomeric/tethered orphan receptors that include steroidogenic factor (SF-1), ROR, Reverb, and small heterodimeric partner (SHP).

Although all nuclear receptors regulate gene expression, among the four classes there are subtle differences in the biochemical mechanisms. Nuclear receptors can be found in either the cytoplasm or nucleus. For some receptors (i.e. GR, ER), unliganded nuclear receptors are bound to heat shock proteins which makes them unable to activate transcription. In the presence of
their ligand, NRs dissociate from the heat shock protein and are able to homodimerize or heterodimerize with RXR, which allows the NRs to pass through the nuclear pores to bind to their response elements that exists as half-sites separated by variable length nucleotide spacers between direct or inverted half-site repeats in the genome. Once the NR binds to DNA, this NR/DNA complex then recruits additional proteins which are involved in activating transcription of target genes.

Figure 1.1 Human Nuclear Receptors Classes (Olefsky 2001)

1.1.2.2 Nomenclature

In the past decade, over 300 genes were identified belonging to the nuclear receptor superfamily, leading to the development of a system to identify nuclear receptors by classification and nomenclature. This system became inefficient because there were several names for the same gene, and was especially problematic for the orphan receptors since they were described by their sequence, not by their function, at the time of their discovery (Enmark and Gustafsson 1996). After being discussed at the Seventh International CBT Symposium on “Nuclear Orphan Receptors” in Huddinge, Sweden, in 1995, it was decided that the system had
to be changed because it was too confusing. For that reason, four scientists formed together to form a committee for the nomenclature of nuclear receptors to incorporate nuclear receptors genes already identified, but to be flexible enough to allow newly identified nuclear receptors to be added. In 1999, the new nomenclature was released and it is based on the evolution of the two most highly conserved domains of nuclear receptors, which are the DNA binding domain and the ligand binding domain, and uses molecular phylogeny to connect and classify all known nuclear receptor sequences (Laudet 1999) corresponding to receptor function. For example, the estrogen receptor binds to estrogen, which is a hormone that is involved in reproduction. Hence, the main function of this receptor involves reproductive development. This system yields six subfamilies and 26 groups of receptors. Each receptor/gene name is denoted by the letters ‘NR’ and a three digit identifier containing an Arabic number such as 1, 2, 3, etc. for the subfamily, a capital letter for the group, and another Arabic number for the gene. The term isoform is used to describe the different gene products originating from the same gene due to alternative promoters, alternative splicing, or alternative starts of translation. Isoforms are designated by a lowercase letter following the last Arabic number. All the unusual receptors that contain either the DNA binding domain or the ligand binding domain are grouped separately as subfamily 0 (Table 1.2). For example, the liver x receptor (LXR) β which belongs to subfamily 1, group H, and is the second identified in that group, is named NR1H2. When a manuscript is written dealing with NRs, it is recommended that the NR’s be identified by the official name at least once in the Summary and the Introduction of the manuscript. Once the official name is established, (e.g. “this paper describes LXRβ (NR1H2), a member of nuclear receptor superfamily”) then the author may use the trivial at this point. When a new NR sequence is identified, researchers are requested to send the amino acid sequence of the newly discovered NR to Vince Laudet (Laudet 1999).
1.1.3 Nuclear Receptor Protein Structure

Nuclear receptors share a common structural organization which consists of five structural domains (A-F) (Krust, Green et al. 1986) (Figure 1.2). The N-terminal regulatory domain (A/B domain) may contain the activation function 1 (AF-1) domain, whose action is independent of the presence of ligand (Novac and Heinzel 2004). The transcriptional activation activity of AF-1 is normally very weak. The DNA binding domain (DBD, C domain) consists of two zinc fingers that bind to the corresponding response element. The hinge region (D domain) is thought to be a flexible region that connects the DBD with the LBD and varies in length between various nuclear receptors. The ligand binding domain (LBD) (E domain) is responsible for ligand binding and dimerization of receptor if it is a dimer. The LBD also interacts with coactivator and corepressor proteins usually via LxxLL motifs. The C-terminal domain (F domain) is a region that contains the activation function 2 (AF-2) which stimulates transcription once the ligand binds.

The three-dimensional (3D) structure of proteins is obtained through a combination of several techniques that consist of x-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy. The 3D structure of a NR DBD was obtained from GR-DBD and ER-DBD using NMR spectroscopy (Luisi, Xu et al. 1991; Schwabe, Chapman et al. 1993; Steinmetz, Renaud et al. 2001). So far, structures of NR LBD have been shown for VDR, AR, ER, PPAR, LXR and RXR (Wurtz, Bourguet et al. 1996; Uppenberg, Svensson et al. 1998; Norman, Adams et al. 1999; Färnegårdh, Bonn et al. 2003). These findings gave insight on the actions of NR such as the molecular dynamics of ligand binding, coactivator interaction, and transactivation which allowed for the design of synthetic NR ligands to modulate these actions (Renaud and Moras 2000; Son and Lee 2009). Recently, the first intact structure of the nuclear receptor on multiple domains such as DNA, ligands, and coactivator peptides was described between PPARδ and
RXR as a heterodimer bound to DNA while other structural studies focused on isolated DNA (Chandra, Huang et al. 2008).

1.1.4 Coactivators and Corepressors

The functional activity of a NR is not solely regulated by hormones and other endogenous lipophilic ligands, because they also form a complex with regulatory proteins including corepressors and coactivators. When these NRs are not bound by ligand, they are complexed with corepressors. These corepressors include receptor interacting protein 140 (RIP140), nuclear receptor corepressor (NCoR), and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) which inhibit nuclear receptors transcriptional activation activity often through recruitment of histone deacetylases and DNA methyltransferases on a target gene’s promoter. These enzymatic activities promote the association of proteins that are involved in maintaining heterochromatin and silenced regions of DNA (Metivier, Leray et al. 2003). The NR becomes activated when bound by ligand, resulting in dissociation of the corepressors and in recruitment of coactivators along with other proteins that are responsible for transcription, such as RNA polymerase II. Coactivator recruitment is an important component of NR signaling pathways (Nolte, Wisely et al. 1998). The activities of coactivators include chromatin remodeling and modifications of histones including ubiquination, sumoylation, acetylation, methylation, and phosphorylation, that allow transcription to occur. For example, the dynamic performance and decision processes of eukaryotic cells are controlled by post-translational modifications, such as protein phosphorylation. Phosphorylation of nuclear receptors can play a major role in their regulation and function (Sun, Montana et al. 2007). Nuclear receptors can be phosphorylated in their N-terminal activation domains (AF-1), the DNA binding domain, and the ligand binding
### Table 1.2 A Proposed Nomenclature for Nuclear Receptors (Laudet 1999)

<table>
<thead>
<tr>
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<td>KNI, Knirps</td>
<td>X13331</td>
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<td>X14153</td>
</tr>
<tr>
<td></td>
<td>NR0A3</td>
<td>EAGON, Embryonic gonad, EAGLE</td>
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<td>ODR7</td>
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<td>Trithorax</td>
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<td>NR0B1</td>
<td>DAX1, AHCH</td>
<td>S74720</td>
</tr>
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<td></td>
<td>NR0B2</td>
<td>SHP</td>
<td>L76571</td>
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Figure 1.2 General structures of NRs (de Lera, Bourguet et al. 2007).

Phosphorylation of nuclear receptors plays a role in the recruitment of coactivators and transcriptional machinery and therefore activation and/or repression of transcription, as well as in nuclear receptor degradation (Rochette-Egly 2003).

1.1.5 Nuclear Export

A number of the nuclear receptors are known to shuttle between the cytoplasmic and nuclear compartments. Nuclear export of the respective nuclear receptors occurs through nuclear pore complexes (NPCs) but all of the mechanisms of export are not known (Mingot, Bohnsack et al. 2004; Pemberton and Paschal 2005). Nuclear receptors must localize in the nucleus to perform their functions as transcription factors. The intranuclear concentration of most nuclear proteins is maintained by a balance of continuous nuclear import and export. Therefore, regulation of both nuclear import and export are important regulatory checkpoints for nuclear receptor functions.
There is no common pathway for nuclear receptor export; however, nuclear export often occurs through karyopherin-mediated mechanisms. Many nuclear proteins are exported through the karyopherin chromosome region maintenance (Crm-1) which binds to leucine-rich nuclear export sequences (NES) in the cargo protein and RanGTP (Fornerod, Ohno et al. 1997; Tsay, Lin et al. 1999; Lo, Gay et al. 2004). It has been shown that ER and ligand bound VDR are exported via Crm-1 (Lee and Bai 2002; Prufer and Barsony 2002; Nonclercq, Journe et al. 2007). Export via Crm-1 can be inhibited by leptomycin B (Kudo, Matsumori et al. 1999). In addition to Crm-1, another karyopherin transporting protein from the nucleus, Exportin 7, was identified. Exportin 7 binds and exports 14-3-3ζ out of the nucleus (Gorlich and Kutay 1999).

Interestingly, 14-3-3 has been shown to regulate the trafficking of nuclear receptors such as GR and ChREBP (Kino, Souvatzoglou et al. 2003; Sakiyama, Wynn et al. 2008)

Figure 1.3 Schematic of a Model for Directional Nucleocytoplasmic Transport Import or Export of Receptor-Cargo Complexes through Nuclear Pore Complex (Fornerod, Ohno et al. 1997).
1.2 Liver X Receptors

Liver X Receptors (LXRs) are ligand-induced transcription factors that belong to the Type II nuclear receptor superfamily. LXR subfamily consists of two subtypes called LXRα (NR1H3) and LXRβ (NR1H2). Initially, they were discovered by screening a rat and human liver cDNA library and were classified as nuclear orphan receptors since their ligand was unknown (Apfel, Benbrook et al. 1994; Song, Kokontis et al. 1994; Willy, Umesono et al. 1995). The LXR proteins each consist of four domains: 1) N-terminal ligand-independent, AF-1 domain, which may stimulate transcription in the absence of ligand, 2) DBD, which contains two zinc fingers, 3) hinge region 4) LBD, which is required for ligand binding and receptor dimerization, and 4) ligand-dependent, AF-2 domain, which stimulates transcription in response to ligand binding. Upon ligand activation, both receptors form obligate heterodimers with RXR to regulate transcription through binding to LXR response elements (LXRE) in the promoter region of target genes (Figure 1.4). The LXRE consists of two AGGGTCA sites separated by four bases called direct repeat-4 (DR-4). The LXR/RXR interaction is considered a “permissive heterodimer” because LXR/RXR interaction may be activated by the ligand of either partner in an independent manner (Willy, Umesono et al. 1995). In the absence of ligands, LXR/RXR heterodimers recruit complexes consisting of corepressors (NCoR/SMRT) and prevent transcription (Brendel, Schoonjans et al. 2002; Hu, Li et al. 2003; Phelan, Weaver et al. 2008) (Figure 1.3). Whereas when ligand is present, the corepressors dissociate allowing coactivators (SRC1 and p300) to be recruited along with other transcriptional machinery (Wiebel, Steffensen et al. 1999).

LXRs regulate cholesterol transport in the liver and macrophages and under normal circumstances prevent atherosclerosis (Repa and Mangelsdorf 2002; Tangirala, Bischoff et al. 2002; Terasaka, Hiroshima et al. 2003; Crestani, De Fabiani et al. 2004). Synthetic LXR ligands potentially could be used to treat diseases such as atherosclerosis (Terasaka, Hiroshima et al. 2003).
2003), but their lipogenic effect in the liver causes hypertriglyceridemia, an undesirable side effect (Plosch, Kok et al. 2002).

LXR\(_{\alpha}\) and LXR\(\beta\) share ~78% identity of their amino sequences in both their DBD and LBD (Ulven, Dalen et al. 2005). They are activated by oxidized cholesterol derivatives or oxysterols such as 22-(R) hydroxycholesterol and 24-(S) hydroxycholesterol, which are naturally occurring, and by synthetic ligands T0901317 and GW3965 (Willy, Umesono et al. 1995; Janowski, Willy et al. 1996; Peet, Janowski et al. 1998; Urban, Cavazos et al. 2000) (Figure 1.4). In contrast to oxysterols being able to activate LXRs, geranyl geranyl pyrophosphate, an intermediate of cholesterol biosynthesis pathway, inhibits both LXR isoforms by acting as an antagonist. The antagonist is able to inhibit the interaction between LXRs and coactivators (Forman, Ruan et al. 1997; Gan, Kaplan et al. 2001).

**Figure 1.4** The mechanism of transcriptional activity by LXRs (Baranowski 2008).
The LXR/RXR interaction has a crystal structure of LXRα/RXRβ heterodimer. It was identified that histidine 383, histidine 390, and glutamic acid residues 387 in LXRα act as important residues for a strong interaction with glutamic acid 465, glutamic acid 472, alanine 469, in RXRβ forming a salt bridge (Svensson, Ostberg et al. 2003). The RXR subfamily includes three isoforms such as RXR α, β, and γ which also have the same amino acids that play a role in the stability of the LXRα/RXRβ heterodimer. However, LXRβ has a glutamine and

![Image of natural and synthetic ligands](image_url)

**Figure 1.5** Liver X Receptor natural and synthetic ligands (Zaveri, Murphy et al. 2007)

leucine at positions that are analogous to LXRα histidine positions 383 and 390. These differences between LXRα and LXRβ protein sequence may play a role in their unique functions.

LXRs regulate cholesterol transport in the liver, intestine, and macrophages (Repa and Mangelsdorf 2002; Tangirala, Bischoff et al. 2002; Terasaka, Hiroshima et al. 2003; Crestani, De Fabiani et al. 2004) by a process referred to as reverse cholesterol transport (RCT). Although LXRα and LXRβ share 78% similarity in amino acid sequence within both the DBD and LBD, they do have differences in tissue distribution, gene expression, post-translational modifications, and protein-protein interactions. Specifically, LXRα is abundantly expressed in the liver,
intestine, kidney, spleen, and adipose tissue, whereas LXRβ is ubiquitously expressed at lower levels (Teboul, Enmark et al. 1995; Willy, Umesono et al. 1995; Repa, Li et al. 2007).

LXRs exert their function by regulating target genes involved in metabolic pathways by binding to DNA sequences in the promoter region of genes (Table 1.3). Even though both LXRα and LXRβ are expressed in liver and macrophages, some genes are selectively activated by LXRα or LXRβ. Under normal circumstances, LXRs induce the expression of cholesterol 7 alpha-hydroxylase (Cyp7A1) via binding to an LXRE present in the promoter of Cyp7A1, which codes for an enzyme that induces the conversion of cholesterol to bile acids then it is removed from the body (Lehmann, Kliever et al. 1997). However, this induction seems to be species specific as LXR suppresses expression of Cyp7A1 in primary human hepatocytes (Chiang, Kimmel et al. 2001; Goodwin, Watson et al. 2003). This repression may be due to the interaction between LXR and small heterodimer partner (SHP), because SHP has a known repressive effect on Cyp7A1 expression (Brendel, Schoonjans et al. 2002; Shang, Pan et al. 2006).

LXRα−/− mice failed to induce Cyp7A1 and caused an accumulation of large amounts of cholesterol esters in the liver (Peet, Janowski et al. 1998; Alberti, Schuster et al. 2001; Quinet, Savio et al. 2006). Also, it has been shown that mice fed a high-fat diet accumulate more cholesterol in both LXRα−/− and LXRαβ−/− than in wild-type LXRβ or LXRβ−/− mice (Korach-Andre, Parini et al. 2010). Both ABCA1 and SREBP-1c expression was less in the liver of LXRα−/− mice than in wild-type controls (LXRβ effect). In addition, LXRα−/− and ApoE−/− mice have shown that LXRβ activation results in more effective gene activation in the periphery, such as in macrophages, than in the liver. These results showed that LXRβ activation did not cause hypertriglyceridemia (Schuster, Parini et al. 2002; Lund, Peterson et al. 2006; Quinet, Savio et al. 2006; Bradley, Hong et al. 2007).
Additional studies have shown that LXRs regulate genes in the family of ABC transmembrane lipid transporters including ABCA1 (Costet, Luo et al. 2000; Repa, Turley et al. 2000), ABCG1 (Venkateswaran, Repa et al. 2000; Kennedy, Venkateswaran et al. 2001; Sabol, Brewer et al. 2005), ABCG5 and ABCG8 (Repa, Berge et al. 2002; Yu, York et al. 2003). These transporters are responsible for exporting lipids and cholesterol from peripheral tissues to the liver in a process known as reverse cholesterol transport (RCT) (Costet, Luo et al. 2000; Repa, Turley et al. 2000; Venkateswaran, Repa et al. 2000; Kennedy, Venkateswaran et al. 2001; Sabol, Brewer et al. 2005; Zelcer and Tontonoz 2006). When ABCG5 and ABCG8 are expressed, they lead to decreased cholesterol absorption from the intestinal lumen and increased

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Target Tissue</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP7A1</td>
<td>liver</td>
<td>bile acid synthesis</td>
</tr>
<tr>
<td>ABCA1</td>
<td>macrophage, intestine</td>
<td>cholesterol efflux</td>
</tr>
<tr>
<td>ABCG5/8</td>
<td>liver, intestine</td>
<td>sterol transport</td>
</tr>
<tr>
<td>ABCG1</td>
<td>macrophage</td>
<td>cholesterol efflux</td>
</tr>
<tr>
<td>APOE</td>
<td>macrophage, adipocyte</td>
<td>component of lipoprotein</td>
</tr>
<tr>
<td>LPL</td>
<td>liver, macrophage</td>
<td>Triglyceride hydrolysis</td>
</tr>
<tr>
<td>FAS</td>
<td>liver, adipocyte</td>
<td>cholesterol metabolism</td>
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<td>SREBP1C</td>
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<td>fatty acid synthesis</td>
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<tr>
<td>LXRα</td>
<td>macrophage</td>
<td>cholesterol sensor</td>
</tr>
</tbody>
</table>
transport of cholesterol from the liver to the bile duct (Repa, Berge et al. 2002; Yu, York et al.

Apolipoproteins serve as cholesterol acceptors in lipoprotein particles and are induced by

LXRs, such as ApoE (Laffitte, Repa et al. 2001; Mak, Laffitte et al. 2002).

LXRα and LXRβ have been shown to undergo differential SUMOylation where LXRβ

was SUMO1 conjugated by PIAS1, whereas LXRα was SUMO2/3 conjugated by HDAC4 in

brain astrocytes (Lee, Park et al. 2009). In addition, LXRα and LXRβ are both phosphoproteins

(Chen, Pace et al. 1999; Mo, Fang et al. 2002). Phosphorylation of LXRα affects its function:

LXRα transcriptional activity decreased upon activation of Protein Kinase C (PKC) in Cos-1,

HEK293T, and HepG2 cells (Delvecchio, Bilan et al. 2007), and the SREBP-1c promoter was

inhibited by Protein Kinase A (PKA) through LXRα in the liver (Yamamoto, Shimano et al.

2007). Phosphorylation of LXRβ by ALK-1 induces cytoplasmic localization (Mo, Fang et al.

2002), but no data are available about the functional consequences of phosphorylation of LXRβ

by either PKA or PKC. In addition, tissue-selective regulation of ABCA1, ABCG1, SREBP-1C,

and LPL expression by phosphorylated LXRα was shown in macrophages (Torra, Ismaili et al.

2008).

The LXRs have several unique protein-protein interactions which may play a role in the

nonredundant functions of LXRs. SHP interacts with LXRα in an AF-2 dependent manner

(Brendel, Schoonjans et al. 2002). It has been shown that both LXRα and LXRβ interact with

the C-terminal domain of NCoR. In addition, it was shown that the interaction was isoform

selective since LXRα has a stronger interaction with NCoR while LXRβ did not suggesting that

the two isoforms recruit repressors differently (Hu, Li et al. 2003; Phelan, Weaver et al. 2008).

LXRs are assumed to undergo nuclear export but the export receptor that is responsible is

unknown. It has been shown that unliganded and ligand-bound LXRα and LXRβ are slowly
exported from the nucleus but while in the presence of the LXR antagonist, geranyl geranyl pyrophosphate (ggPP), there was faster nuclear export of LXR (Prufer and Boudreaux 2007).

Although LXR have been extensively studied, the mechanism of tissue and LXR-isotype selectivity is poorly understood. Taken together these findings indicate a role for LXR in promoting an increased reverse cholesterol transport (RCT), decreased dietary cholesterol uptake as well as increased cholesterol efflux. These features are highly beneficial in preventing metabolic disorders but if characterized it might suggest LXR being used as therapeutic targets.

1.3 14-3-3 Proteins

The 14-3-3 proteins are a family of molecules that regulate intracellular signal transduction events within all eukaryotic cells. These 30kDa α-helical molecules consist of seven isotypes (β, γ, ε, σ, ζ, τ, and η) in mammals, 15 isoforms in Arabidopsis and two each in Drosophila melanogaster and Caenorhabditis elegans (Rittinger, Budman et al. 1999; Fu, Subramanian et al. 2000; Tzivion, Shen et al. 2001). 14-3-3 proteins are able to form dimers. These dimeric 14-3-3 proteins are ubiquitously expressed and play a role in a wide variety of signal transduction pathways including, the mitogen activated protein kinase pathway (MAPK) pathway. In the MAPK pathway, Raf links activated cell surface receptors to the classical MAPK, extracellular signal-regulated kinase (ERK) 1 and 2, and MAPK/ERK. In addition to Raf, mitotic phosphatase Cdc25C/B regulates entry from G2 into mitosis and apoptosis through the BH3 domain-only pro-apoptotic protein (Fanger, Widmann et al. 1998; Rittinger, Budman et al. 1999; Fu, Subramanian et al. 2000; Tzivion, Shen et al. 2001). 14-3-3 participates in regulating the subcellular localization and function of both Raf and Cdc25C/B (Brunet, Kanai et al. 2002).

14-3-3 regulates cellular processes by affecting the protein’s localization (Muslin, Tanner et al. 1996). In most cases, 14-3-3 binding sequesters the phosphorylated target protein in a particular subcellular compartment, and dephosphorylation of the target protein releases it from
14-3-3 and makes it available to signaling pathways. This mechanism of regulation contributes to the nuclear retention of proteins including human telomerase reverse transcriptase (TERT), thioredoxin-like protein 2 (Txl-2), and checkpoint kinase (Chk1) (Tang, Suen et al. 1998; Seimiya, Sawada et al. 2000; Jiang, Pereira et al. 2003).

14-3-3 proteins interact with target proteins that are phosphorylated but they can bind to those that are not phosphorylated as well. 14-3-3 proteins form homo- and heterodimeric cup-shaped structures (Liu, Bienkowska et al. 1995; Xiao, Smerdon et al. 1995). These structures allow 14-3-3 proteins to bind to a large number of phosphorylated proteins through one or more short phosphoserine/threonine containing sequence motifs in target proteins (Muslin, Tanner et al. 1996; Yaffe, Rittinger et al. 1997). 14-3-3 proteins recognize sequences R (S/Ar)XpSXP and R X(Ar/S)XpSXP, in which ‘Ar’ denotes an aromatic residue (Yaffe, Rittinger et al. 1997). However, a few of the 14-3-3 binding peptides have been shown with sequences that deviate from these motifs or do not require phosphorylation. For example, binding of 14-3-3 to proteins like R18, a Raf derived peptide (Fu, Subramanian et al. 2000), and carbohydrate response element-binding protein (ChREBP) does not require a phosphorylated residue (Li, Chen et al. 2008).

14-3-3σ, also known as stratifin, has been widely studied because of its affiliation with cancer (Hermeking 2003). 14-3-3σ is a target gene of p53 and upregulation of 14-3-3σ leads to cell cycle arrest (Hermeking, Lengauer et al. 1997). Also, it has been shown that 14-3-3σ interacts with members of the nuclear receptor superfamily, the glucocorticoid receptor (GR) α and the androgen receptor (AR) (Widen, Zilliacus et al. 2000; Quayle and Sadar 2007). 14-3-3σ was shown to increase the transcriptional activity of AR in the absence of ligand and act as a negative regulator of GRα signaling (Widen, Zilliacus et al. 2000; Kino, Souvatzoglou et al. 2003). These data together led us to hypothesize that interaction of 14-3-3σ with LXRs may
play a role in functions of LXR$s$. Therefore, we explored whether 14-3-3σ interacts with and affects the functions of LXR$\alpha$ and LXR$\beta$.

1.4 Cell Lines

1.4.1 HepG2 Cells

HepG2 cells are a human liver carcinoma cell line that was derived from a 15 year old Caucasian American male and have an epithelial morphology. HepG2 cells are used routinely for a variety of biochemical and cell biological studies which include liver specific metabolic functions such as cholesterol synthesis, cholesterol transport, cholesterol uptake and cholesterol secretion (Erickson and Fielding 1986; Javitt 1990; Pandak, Stravitz et al. 1996; Izem, Rassart et al. 1998). HepG2 cells can be propagated in cell culture in contrast to primary liver cells which cannot.

1.4.2 HEK 293 Cells

Human Embryonic Kidney 293 cells are a cell line that was derived from an aborted fetus then transformed by adenovirus 5 DNA. The number 293 was originated from Frank Graham’s numbering system of his experiments so the original clone was the product of his 293rd experiment. HEK 293 cells are very easy to grow and transfect and have been widely used in cell biology research. Also, they are used by the biotechnology industry to produce therapeutic proteins and viruses for gene therapy.

1.4.3 Cos-7 Cells

Cos (CV-1 (simian) in origin, and carrying the SV40 genetic material) is a cell line that was derived from kidney cells of the African green monkey which has a form of the SV40 genome obtained by using a constitutively active CV-1 (Jensen, Girardi et al. 1964). The SV40 genome can produce T antigen but it cannot replicate its genome (Gluzman 1981). There are
two different types of Cos cells which are Cos-1 and Cos-7. They are used because they are easily transfected and can produce recombinant proteins for studies in biology.

**Research Focus:** Overall, the goal of LXR research is to explore a mechanism which will allow LXRs to be used as therapeutic targets for metabolic disorders. However, possible side effects may arise and can possibly be avoided if LXRs are characterized more extensively to promote selective activation of genes. This could be achieved by selective activation of an LXR isoform. Specifically, our lab studies LXR isotype selectivity which includes selective protein-protein interactions. In this document, we will show that 14-3-3ζ binds to LXRβ not LXRα, the region that 14-3-3ζ binds to LXRβ, how this interaction may affect the ability of LXRβ to bind to DNA, the effect that 14-3-3ζ has on LXR regulated genes, and how nuclear export of LXRs requires energy but not Crm-1.
CHAPTER 2
MATERIALS AND METHODS

2.1 Cells and Expression Constructs

Cyan fluorescent protein tagged (CFP)-LXRα, CFP-LXRβ, and CFP-RXR were obtained, generated, and verified as described earlier (Prüfer, Racz et al. 2000; Prüfer 2007). The C-terminal truncated LXRβ mutants were created using QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA). We inserted stop codons into the LXRβ sequence to replace the codons encoding either amino acid 106 or 156, creating truncated LXRβ containing either the first 105 amino acids (105CFP- LXRβ) or the first 155 amino acids (155CFP- LXRβ). In all constructs the mutations were verified by DNA sequencing (ABIPrism, Applied Biosystems, CA). Also, we cloned the coding sequences of 14-3-3σ (Harvard Institute of Proteomics, MA), LXRα, and LXRβ into a vector for E. coli expression with an N-terminal glutathione S transferase (GST) fusion (pDEST 15, Invitrogen, Carlsbad, CA) using TOPO cloning and the Gateway system (Invitrogen, Carlsbad, CA). We expressed GST-fusion proteins in E. coli and extracted them according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). In addition, we cloned the coding sequence of 14-3-3σ into both the pDNR-CMV and the CFP vector using the In-Fusion system (Clontech, Mountain View, CA).

Cos-7 and human embryonic kidney (HEK)293 cells were obtained from ATCC (Manassas, VA) and grown as described previously (Prüfer, Racz et al. 2000; Prüfer 2007). Briefly, cell cultures were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 2 mM glutamine (Invitrogen, Carlsbad, CA), and 0.1 mg/ml gentamicin (Invitrogen, Carlsbad, CA).
2.2 Expression and Purification of Proteins

Glutathione S transferase tagged (GST)-LXRα, GST-LXRβ, GST-14-3-3σ (GST fusion proteins) and GST were expressed in BL21 *E. coli*. The bacterial pellets were lysed in lysis buffer [1mg/ml of lysozyme in buffer A (20mM HEPES, pH 7.9, 1mM EDTA, 0.5% Nonidet P-40, 0.1% bovine serum albumin, 10% glycerol), supplemented with protease inhibitors and dithiothreitol (Yasmin, Williams et al. 2005) and phosphatase inhibitors (1mM sodium vanadate and 1mM sodium molybdate)]. Cells were lysed by multiple freeze-thaw cycles followed by sonication. Then, samples of the lysates were run on a 7.5% SDS-PAGE gel together with a BSA standard. The amount of expressed GST fusion proteins was estimated, and approximately 30 µg GST fusion proteins/experiment were bound to glutathione agarose beads.

2.3 *In vitro* Binding Assay

HEK293 cells were transfected with CFP-LXRα, wild-type and C-terminal truncated CFP-LXRβ, and CFP-14-3-3σ using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 hours, the cells were scraped, washed with cold phosphate buffer saline (PBS), resuspended in supplemented buffer A (Yasmin, Williams et al. 2005), and subjected to one freeze/thaw cycle. The presence of expressed protein in the supernatants was verified by Western Blot analysis with the GFP monoclonal antibody, JL-8 (Clontech, Mountain View, CA).

Cell extracts containing CFP-LXRα, wild-type CFP-LXRβ or truncated CFP-LXRβ were combined with either GST-14-3-3σ to test interaction or with GST to serve as a negative control. Cell extracts containing CFP-14-3-3σ were combined with GST, GST-LXRα, or GST-LXRβ. All GST pull downs were rotated for three hours at 4°C, washed three times with supplemented buffer A (Yasmin, Williams et al. 2005), and then subjected to Western Blot analysis with the GFP monoclonal antibody, JL-8. All experiments were repeated at least three times.
2.4 Transactivation Assays

Cos-7 cells were subcultured into 96-well plates (Nalge Nunc Int., Naperville, IL). Transcriptional activities of LXRα and LXRβ with and without 14-3-3σ were tested by co-transfection experiments. In all experiments the amount of DNA transfected was normalized by co-transfection of an empty vector (pDNR-CMV, Clontech). Cells were transfected with combinations of expression plasmids (0.5 μg /8 wells), LXRE luciferase reporter (0.5 μg /8 wells; gift from Dr. Auwerx, CNRS/LGME-INSERM, Illkirch, France), and the Renilla-luciferase control plasmid pHRLTK (Promega; 0.1 μg /8 wells) using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA). Six hours after transfection, media was changed to media containing 5% FBS without lipoproteins (Intracel, Frederick, MD) to deplete cells of LXR ligands. Cells were treated with either vehicle (0.1% DMSO and 0.1% ethanol) or 0.1 μM 9-cis retinoic acid combined with 1 μM T090317 (EMD, San Diego, CA) 24 h after transfection.

Twenty-four hours after treatment, cells were lysed on the culture plate using passive lysis buffer (Promega, Madison, WI), after which the samples were frozen and thawed for better lysis. Luciferase activities were measured using Dual-Luciferase assay reagents (Promega, Madison, WI) in a Fluostar Optima with two injectors (BMG Labtech, Offenburg Germany). Luminescence data were normalized to Renilla luciferase values and expressed as fold induction relative to vehicle-treated controls. Data are average from 8 data points and are presented as the mean ±1 S.E.

2.5 Electrophoretic Mobility Shift Assay (EMSA)

We used histidine-tagged LXRβ and RXR for the EMSA. The oligos contained the LXRE sequence in the ABCA1 promoter (MWG Biotech, High Point, NC):

Forward: 5’GATCTTTTTGACCAGATAGTAACCTCTGCGCG 3’;
Reverse: 5’GATCCCGCGAGGTTACTATCGGTCAAAA 3’.
The oligos were annealed and then end-labeled with biotin. In the binding reactions, 500ng of each purified protein was used together with 2 pM biotinylated oligos. In samples where LXR and RXR are present, LXR and RXR were mixed in a 1:1 ratio and allowed to dimerize for 10 min prior to use. The binding reactions were incubated at room temperature for 20 min along with GST and GST-14-3-3σ. The binding reactions were loaded and electrophoresed on a pre-cast 5% polyacrylamide TBE gel (Bio-Rad, Hercules,CA) and then transferred to a Biodyne B Pre-cut modified nylon membranes, 0.45µm (Pierce Biotechnology, Thermo Scientific, Rockford, IL). The membrane was incubated following the Light Shift Chemiluminescent EMSA Kit protocol (Pierce Biotechnology, Thermo Scientific, Rockford, IL).

2.6 Microscopy

Cos-7 cells were plated on chambered cover glasses (Nalge Nunc Int., Naperville, IL) and transfected with either YFP-LXRβ or CFP-14-3-3σ using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After transient transfections, cells were used for microscopy within 48 h. For imaging experiments, cells expressing YFP-LXRβ or CFP-14-3-3σ either alone or together were cultured for 18 h in media containing 5% FBS without lipoproteins (Intracel, Frederick, MD) prior to the experiment to deplete cells of the ligands for LXR. For a subset of imaging experiments, cells were treated with vehicle (0.2% DMSO) or a combination of 100nM 9-cis-retinoic acid (Sigma, St. Louis, MO) and 1mM T09 (EMD, San Diego, CA) along with either 1mM 12-O-tetradecanoylphorbol-13-acetate (TPA, a protein kinase C activator) or 100mM forskolin (a protein kinase A activator) for 24 hours after transfection.

Images were collected from an Olympus IX81 fluorescence microscope using a 40×/0.6 NA objective and a digital CCD camera (Hamamatsu Photonics, Japan). The Yellow GFP BP filter (exciter: HQ500/20, emitter HQ535/30, beamsplitter Q515Ip; Chroma Technology Corp, Rockingham, VT) was used for detection of YFP. The Cyan GFP v2 filter (exciter: D436/20,
emitter D480/40, beamsplitter 455dcIp; Chroma Technology Corp.) was used for detection of CFP. Nuclear and cytoplasmic fluorescence intensities were measured using IPLab software (Scanalytics Inc., Fairfax, VA) in each 50–100 cells expressing either YFP-LXRβ or CFP-14-3-3σ either alone or in combination with each other. Cells were randomly chosen by imaging several areas of fluorescing cells.

2.7 Permeabilization Experiments

Nuclear export assays were performed as described previously (Prufer and Barsony 2002). HEK293 cells were plated onto chamber slides coated with gelatin. Twenty-four hours later the cells were transfected with plasmids expressing either YFP-LXRα or YFP-LXRβ using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, cells were treated with vehicle (0.2% DMSO), leptomycin B, or antagonist (1µM geranyl geranyl pyrophosphate (ggPP, Sigma, St. Louis, MO) for one hour at 37°C. The above treatments were present both during permeabilization, during washing steps and for one hour at 37°C (or 4°C as indicated) after permeabilization. Transport buffer (TB) contained both protease inhibitors and cyclohexamide. Rabbit reticulocyte lysate was added to the TB where indicated. Cells in one chamber were treated for 10 minutes on ice with TB containing 50µg/mL digitonin. Nuclear and cytoplasmic fluorescence intensities were measured using IPLab software (Scanalytics Inc., Fairfax, VA) in each 50–100 cells expressing either YFP-LXRβ or CFP-14-3-3σ either alone or in combination. Cells were randomly chosen by imaging several areas of fluorescing cells.
CHAPTER 3

14-3-3σ INTERACTS WITH LIVER X RECEPTOR β BUT NOT LIVER X RECEPTORα

3.1 Introduction

Liver X Receptors (LXR) are transcription factors that belong to the Type II Nuclear Receptor family. These receptors include two subtypes, LXRα and LXRβ, which are activated by oxysterols (Willy, Umesono et al. 1995; Janowski, Willy et al. 1996; Peet, Janowski et al. 1998). LXR regulates transcription of genes for proteins that play key roles in cholesterol metabolism and transport such as the ATP-binding cassette (ABC) A1 transporter (Venkateswaran, Laffitte et al. 2000; Chawla, Boisvert et al. 2001), and for lipogenesis such as the steroid response element binding protein (SREBP)-1c (Repa, Liang et al. 2000; Cagen, Deng et al. 2005). LXR regulates cholesterol transport in the liver and macrophages, and under normal circumstances prevent atherosclerosis (Repa and Mangelsdorf 2002; Tangirala, Bischoff et al. 2002; Terasaka, Hiroshima et al. 2003; Crestani, De Fabiani et al. 2004). Synthetic LXR ligands could be used to treat diseases such as atherosclerosis (Terasaka, Hiroshima et al. 2003), but their lipogenic effect in the liver causes hypertriglyceridemia (Plosch, Kok et al. 2002). LXRα and LXRβ share 78% identity in DNA and ligand-binding domains. LXRα is expressed at high levels in liver, intestine, adipose tissue, and macrophages (Willy, Umesono et al. 1995), whereas LXRβ is expressed ubiquitously (Teboul, Enmark et al. 1995). Knock-out studies have shown that LXRβ more effectively activates genes in peripheral cells, such as macrophages, than in the liver (Quinet, Savio et al. 2006). The mechanism of this tissue and LXR-isotype selectivity is poorly understood, but if characterized it might suggest therapeutic targets for metabolic disorders.
The 14-3-3 proteins are a highly conserved family that regulates intracellular signal transduction events within all eukaryotic cells. These 30kDa α-helical molecules consist of seven isotypes (β, γ, ε, σ, ζ, τ, and η) in mammals, 15 isoforms in Arabidopsis and two each in Drosophila melanogaster and Caenorhabditis elegans (Rittinger, Budman et al. 1999; Fu, Subramanian et al. 2000; Tzivion, Shen et al. 2001). Dimeric 14-3-3 proteins are ubiquitously expressed and play a role in a wide variety of signal transduction pathways including, the MAPK pathway. Raf links activated cell surface receptors to the classical mitogen-activated protein kinase pathway (MAPK), extracellular signal-regulated kinase (ERK) 1 and 2, and MAPK/ERK. Mitotic phosphatase Cdc25C/B regulates entry from G2 into mitosis and apoptosis through the BH3 domain-only pro-apoptotic protein (Fanger, Widmann et al. 1998; Rittinger, Budman et al. 1999; Fu, Subramanian et al. 2000; Tzivion, Shen et al. 2001). 14-3-3 participates in regulating the subcellular localization and function of both Raf and Cdc25C/B (Brunet, Kanai et al. 2002).

14-3-3 regulates cellular processes by modulating protein localization (Muslin, Tanner et al. 1996). In most cases, 14-3-3 binding sequesters the phosphorylated target protein in a particular subcellular compartment, and dephosphorylation of the target protein releases it from 14-3-3 and makes it available to signaling pathways. This mechanism of regulation contributes to the nuclear retention of proteins including human telomerase reverse transcriptase (TERT), thioredoxin-like protein 2 (Txl-2), and checkpoint kinase (Chk1) (Tang, Suen et al. 1998; Seimiya, Sawada et al. 2000; Jiang, Pereira et al. 2003).

14-3-3 proteins interact with target proteins that are phosphorylated but they can bind to those that are not phosphorylated as well. 14-3-3 proteins form homodimeric and heterodimeric cup-shaped structures (Liu, Bienkowska et al. 1995; Xiao, Smerdon et al. 1995). These structures allow 14-3-3 proteins to bind to a large number of phosphorylated proteins through one or more short phosphoserine/threonine containing sequence motifs in target proteins.
(Muslin, Tanner et al. 1996; Yaffe, Rittinger et al. 1997). 14-3-3 proteins recognize the sequences $R(S/Ar)XpSXP$ and $R X(Ar/S)XpSXP$, in which ‘Ar’ denotes an aromatic residue (Yaffe, Rittinger et al. 1997). However, a few of the 14-3-3 binding peptides have been shown with sequences that deviate from these motifs or do not require phosphorylation. For example, binding of 14-3-3 to proteins like R18, a Raf derived peptide (Fu, Subramanian et al. 2000), and carbohydrate response element-binding protein (ChREBP) does not require a phosphorylated residue (Li, Chen et al. 2008).

14-3-3σ, also known as stratifin, has been widely studied because of its affiliation with cancer (Hermeking 2003). 14-3-3σ is a target gene of p53 and upregulation 14-3-3σ leads to cell cycle arrest (Hermeking, Lengaüer et al. 1997). Also, it has been shown that 14-3-3σ interacts with members of the nuclear receptor super-family, the glucocorticoid receptor (GR) $\alpha$ and the androgen receptor (AR) (Widen, Zilliacus et al. 2000; Quayle and Sadar 2007). 14-3-3σ was shown to increase the transcriptional activity of AR in the absence of ligand and act as a negative regulator of GR$\alpha$ signaling (Widen, Zilliacus et al. 2000; Kino, Souvatzoglou et al. 2003). These data together led us to hypothesize that interaction of 14-3-3σ with LXRs may play a role in functions of LXRs. Therefore, we explored whether 14-3-3σ interacts with and affects the functions of LXR$\alpha$ and LXRβ.

3.2 Results

3.2.1 14-3-3σ Interacts with LXRβ but Not with LXRα

14-3-3σ was shown to interact with AR and GR (Widen, Zilliacus et al. 2000; Kino, Souvatzoglou et al. 2003). Since AR and GR are nuclear receptors similar to LXRs, we hypothesized that 14-3-3σ may also interact with LXR$\alpha$ and/or LXRβ. We performed GST pulldown assays where either GST, GST-LXR$\alpha$, or GST-LXRβ was incubated with extracts
from HEK 293 cells expressing CFP-14-3-3σ to determine if 14-3-3σ interacts with the LXRα.
The expression of similar amounts of GST, GST-LXRα, GST-LXRβ, and GST-14-3-3σ was verified using Western blot analysis (Figure 3.1). We found that GST-LXRβ but not GST-LXRα interacts with CFP-14-3-3σ (Figure 3.2). As a positive control, we included extracts from cells expressing CFP-RXR, a known binding partner of both LXRα and LXRβ, and incubated these extracts with GST, GST-LXRα, or GST-LXRβ. We found that both GST-LXRα and GST-LXRβ interact with CFP-RXR as expected (Figure 3.3). As a negative control, we included extracts from cells overexpressing CFP, which did not bind GST-LXRα and GST-LXRβ (data not shown). These data show that 14-3-3σ interacts with LXRβ but not LXRα. To confirm that

![Western blot analysis](image)

**Figure 3.1** GST, GST 14-3-3σ, GST-LXRα, and GST-LXRβ are being expressed in *E. coli*. Extracts from bacterial cells expressing GST, GST 14-3-3σ, GST-LXRα, and GST-LXRβ were analyzed by Western blot analysis with the GST antibody.
Figure 3.2 LXRβ but not LXRα interacts with 14-3-3σ
GST constructs were expressed, purified, and bound to glutathione agarose beads (Sigma, St. Louis, MO) at 30 μg/μl beads using standard methods. HEK293 cells were transfected with CFP-14-3-3σ, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were lysed in the presence of phosphatase inhibitors. The GST pull down assays were performed as described previously (Yasmin, Williams et al. 2005) in the presence of phosphatase inhibitors. After 3 hour incubation at 4°C, beads were washed and denatured in sample buffer containing 5% mercaptoethanol. Samples were separated using SDS-PAGE, and membranes were immunoblotted with GFP antibody (JL-8, Clontech). Lane 1: GST- LXRβ with CFP-14-3-3σ; Lane 2: GST-LXRα with CFP-14-3-3σ; Lane 3: GST control with CFP-14-3-3σ; Lane 4: input CFP-14-3-3σ (5%).

Figure 3.3 LXRα and LXRβ interact with RXR
GST constructs were expressed, purified, and bound to glutathione agarose beads (Sigma, St. Louis, MO) at 30 μg protein/μl beads using standard methods. HEK293 cells were transfected with CFP-RXR using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were lysed in the presence of phosphatase inhibitors. The GST pull down assays were performed as described previously (Yasmin, Williams et al. 2005) in the presence of phosphatase inhibitors. After 3 hour incubation at 4°C, beads were washed and denatured in sample buffer containing 5% mercaptoethanol. Samples were separated using SDS-PAGE, and membranes were immunoblotted with GFP antibody (JL-8, Clontech). Lane 1: GST-LXRα with CFP-RXR; Lane 2: GST-LXRβ with CFP-RXR; Lane 3: GST control with CFP-RXR; Lane 4: CFP-RXR input (10%).

14-3-3σ interacts with LXRβ, we incubated GST-14-3-3σ together with extracts from cells expressing CFP-LXRβ. We found that GST-14-3-3σ interacts with CFP-LXRβ (Figure 3.4)
3.2.2 14-3-3σ Binds LXRβ in the DNA Binding Domain but 14-3-3σ Does Not Affect LXRβ Binding to DNA

To further explore the interaction between 14-3-3σ and LXRβ, we next determined the region within LXRβ that is important for interaction with 14-3-3σ. We tested binding of extracts from HEK 293 cells expressing various C-terminally truncated CFP-LXRβ to GST-14-3-3σ using GST pulldown assays. Whereas all LXRβ containing the first 155 amino acids (155CFP-LXRβ) bound GST-14-3-3σ, LXRβ containing the first 105 amino acids (105CFP- LXRβ) did not (Figure 3.6). These data show that amino acids 106 to 155 within LXRβ are important for the interaction with 14-3-3σ. Since 14-3-3σ binds LXRβ in the DBD (Figure 3.5), we next determined if this interaction affects LXRβ binding to DNA. We

**Figure 3.4** LXRβ interacts with 14-3-3σGST constructs were expressed, purified, and bound to glutathione agarose beads (Sigma, St. Louis, MO) at 30 μg protein/μl beads using standard methods. HEK293 cells were transfected with CFP-LXRβ using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were lysed in the presence of phosphatase inhibitors. The GST pull down assays were performed as described previously (Yasmin, Williams et al. 2005) in the presence of phosphatase inhibitors. After 3 hour incubation at 4ºC, beads were washed and denatured in sample buffer containing 5% mercaptoethanol. Samples were separated using SDS-PAGE, and membranes were immunoblotted with GFP antibody (JL-8, Clontech). **Lane 1:** GST control with CFP-LXRβ; **Lane 2:** GST-14-3-3σ with CFP-LXRβ; **Lane 3:** empty; **Lane 4:** CFP-LXRβ input (5%).
Figure 3.5 Schematic presentations of C-terminal truncations of LXRβ

performed an Electrophoretic Mobility Shift Assay (EMSA) using a probe encoding an LXR response element within the promoter region of the ABCA1 gene. Purified His-LXRβ was incubated in the presence of purified His-RXR along with various concentrations of GST-14-3-3σ. GST alone was used in the same concentrations as GST-14-3-3σ as a control. Figure 3.7 show that His-LXRβ bound the probe in the presence of His-RXR independently of the concentration of 14-3-3σ. We conclude that 14-3-3σ does not affect binding of LXRβ to DNA.

3.2.3 14-3-3σ Decreases Both the Basal and the Ligand-induced Transcriptional Activity of LXRβ

14-3-3σ was shown to increase the transcriptional activity of AR in the absence of ligand and act as a negative regulator of GRα signaling (Widen, Zilliacus et al. 2000; Kino, Souvatzoglou et al. 2003). We hypothesized that 14-3-3σ has an effect on the function of LXRβ. Therefore we tested dose dependence of the effect of 14-3-3σ on the transcriptional activity of LXRβ. We transfected Cos-7 cells with various ratios of LXRβ to 14-3-3σ expression plasmids and found a dose-dependent effect of 14-3-3σ on both the basal and the ligand-induced transcriptional activity of LXRβ (Figure 3.8). Whereas ligand induced the transcriptional activity of LXRβ both in the absence (4.2-fold) and in the presence of 14-3-3σ (2.8-fold for 1:2 and 1:4
3.3 Discussion

The most important finding of this work is that 14-3-3σ selectively interacts with LXRβ but not with LXRα. This result may explain some of the functional differences between the two LXR isotypes. Knockout of LXRα but not of LXRβ causes fatty liver in mice on a high cholesterol diet (Peet, Janowski et al. 1998). LXRα and LXRβ bind the same ligands and bind to the same promoter DNA sequences. Therefore, the functional dominance of LXRα is likely regulated by differences between the LXR isotypes in yet unidentified mechanisms. One of these mechanisms may be related to the inhibitory effect of 14-3-3σ on the ligand-induced transcriptional activity of LXRβ. It is likely that, selective protein-protein interactions regulate the functions of LXRα and LXRβ. Another example of isotype-selective interaction of 14-3-3σ.
has been shown, previously, for two GR isotypes, GRα and GRβ (Kino, Souvatzoglou et al. 2003). Whereas 14-3-3 interacts with GRα it does not interact with GRβ. Such selective interactions of 14-3-3σ with both LXR and GR isotypes may explain functional differences of otherwise very similar receptor isotypes.

Phosphorylation may not play a role in the interactions of 14-3-3σ and LXRβ. Whereas both forskolin and 12-O-tetradecanoylphorbol-13-acetate (TPA), known activators of PKA and PKC respectively, inhibit the functions of LXRα (Delvecchio, Bilan et al. 2007; Yamamoto, Shimano et al. 2007), we found that forskolin had no effect on LXRβ transcriptional activity.

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**Figure 3.7** 14-3-3σ does not affect LXRβ binding to DNA
Demonstration of LXRβ-DNA interaction between LXRβ and increasing amounts of 14-3-3σ by Electromobility Shift Assay (EMSA). The EMSA was performed using the LXR response element of the ABCA1 promoter as the probe along with His-LXRβ with various concentration of either GST or GST-14-3-3σ in the presence of His-RXR.
There was no additional effect of these activators on the 14-3-3ζ induced inhibition of LXRβ transcriptional activity (Figure 3.9). No apparent 14-3-3 binding sequence motif is present in LXRβ. Other examples for phosphorylation-independent interactions of 14-3-3 proteins are interactions with R18, a Raf derived peptide (Fu, Subramanian et al. 2000), and carbohydrate response element-binding protein (ChREBP) (Li, Chen et al. 2008).

We found that 14-3-3ζ inhibits both basal and ligand-induced transcriptional activity of LXRβ; the fold-induction by ligand was only slightly affected. In contrast, 14-3-3ζ inhibits only the ligand-induced transcriptional activity of GRα. That can be explained by the fact that 14-3-3ζ interacts in a partial ligand-dependent fashion with the ligand binding domain of GRα.

![Figure 3.8](image)

**Figure 3.8** 14-3-3ζ decreases the transcriptional activity of LXRβ
Cos-7 cells were transfected with LXRE luciferase reporter and the Renilla-luciferase control plasmid together with LXRβ expression plasmid together with various ratios of LXRβ and 14-3-3ζ. Cells were treated with a combination of DMSO and ethanol (light bars) or a combination of 1μM T090317 and 100nM 9-cis retinoic acid (dark bars). Data are presented as average of 8 data points’ ±1S.E (% of agonist-induced LXR activity without 14-3-3ζ).
14-3-3 binding inhibits the interaction between forkhead box (FOXO) protein and target DNA by masking the ability FOXO to bind DNA (Silhan, Vacha et al. 2009). In contrast, we have shown that despite the fact that 14-3-3σ interacts with the DNA binding domain of LXRβ, the interaction between 14-3-3σ and LXRβ does not impair DNA binding of LXRβ. The effect of 14-3-3σ on the basal transcriptional activation of LXRβ, therefore, is likely mediated through a ligand-independent interaction of 14-3-3σ with the DNA binding domain of LXRβ. This interaction may also be indirect, since we used cell extracts to explore the interactions between LXRβ and 14-3-3σ. 14-3-3 proteins are known to regulate cellular processes by modulating their target protein’s localization. For example, 14-3-3σ negatively regulated GRα by retaining it in the cytoplasm. In contrast to those findings, we

![Normalized Luciferase Activity](image)

**Figure 3.9** PKA or PKC activators had no affect on the transcriptional activity of LXRβ with or without 14-3-3. Cos-7 cells were transfected with LXRE luciferase reporter and the *Renilla*-luciferase control plasmid together with LXRβ. Cells were treated for 24 hours with either ethanol (V, vehicle) or 1μM T090317 and 100nM 9-cis retinoic acid (A, agonist), combined with either DMSO (light bars), forskolin (100nM; gray bars), a protein kinase A (PKA) activator, or
12-\textit{O}-tetradecanoylphorbol-13-acetate (TPA; 1nM; dark bars), a protein kinase C (PKC) activator. Data are presented as average of 8 data points’ ±1S.E.

found that 14-3-3σ did not affect the localization of LXRβ in the presence or absence of PKA and PKC activators (Figure 3.10). These data further support an unconventional mechanism by which 14-3-3σ interacts with and affects the functions of LXRβ.

\textbf{3.5 Conclusion}

In summary, our results show that 14-3-3σ decreases both the basal and the ligand-induced transcriptional activity of LXRβ but not of LXRα. This interaction between 14-3-3σ and LXRβ requires the DNA binding domain of LXRβ but it does not affect LXRβ binding to DNA. Further studies are needed to test the functional importance of these findings.
**Figure 3.10** 14-3-3σ did not affect the localization of LXRβ. Cos-7 cells were transfected with YFP-LXRβ and/or CFP-14-3-3σ. YFP-LXRβ and CFP-14-3-3σ were transfected separately (A) then co-localization was observed using YFP-LXRβ with CFP-14-3-3σ (B). YFP-LXRβ with CFP-14-3-3σ along with forskolin (100nM), a protein kinase A (PKA) activator (C), and YFP-LXRβ with CFP-14-3-3σ along with 12-O-tetradecanoylphorbol-13-acetate (TPA) (1nM), a protein kinase C (PKC) activator (D).
CHAPTER 4

THE MECHANISM OF NUCLEAR EXPORT OF LIVER X RECEPTOR $\alpha$ AND LIVER X RECEPTOR $\beta$ REQUIRES ENERGY AND IS CRM-1 INDEPENDENT

4.1 Introduction

Liver X receptors are transcription factors that belong to a superfamily of nuclear receptors which regulate cholesterol metabolism. Nuclear receptors are known to shuttle between the cytoplasmic and nuclear compartments (Gorlich and Kutay 1999; Nonclercq, Journe et al. 2007; Grespin, Bonamy et al. 2008). Nuclear receptors localization is controlled by their interactions with the nuclear import and export machinery. Transport of nuclear receptors can be either energy dependent or energy independent.

4.2 Results

We have previously shown that unliganded and ligand-bound LXR$\alpha$ and LXR$\beta$ are slowly exported from the nucleus; but while in the presence of the LXR antagonist, geranyl geranyl pyrophosphate (ggPP), there was faster nuclear export of LXRs (Prufer and Boudreaux 2007). Therefore, we performed digitonin permeabilization experiments using ggPP to further explore the mechanism of nuclear export of LXR$\alpha$ and LXR$\beta$. In these experiments, the cell membrane is permeablized using digitonin permeabilization assays but the nuclear membrane remains intact (Prufer and Barsony 2002). After permeablization of the cell membrane, the presence of cytoplasmic components is lost but these components are added in digitonin permeabilization experiments using rabbit reticulocyte lysate (RRL).

We duplicated the results that LXR$\alpha$ and LXR$\beta$ are quickly exported out of the nucleus in the presence of ggPP (Figure 4.1 and Figure 4.2; top panel). Next, we wanted to determine whether nuclear export of LXR$\alpha$ and LXR$\beta$ requires energy. In order to determine whether export of LXR$\alpha$ and LXR$\beta$ requires energy, we used HEK293 cells expressing YFP-LXR$\alpha$ or YFP-LXR$\beta$. 

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in transport buffer (TB) with RRL that were pretreated with ggP. At 4°C nuclear export was slow while at 37°C nuclear export was increased (Figure 4.1 and Figure 4.2; middle panel).

In addition to these findings, we wanted to determine whether LXRα and LXRβ are exported from nucleus by crm-1, we performed digitonin permeabilization assays at 37°C in the presence of RRL, and leptomycin B (2nM; LMB), an inhibitor of export receptor Crm-1 (Kudo, Matsumori et al. 1999). We found that both LXRα and LXRβ were not exported from the nucleus (Figure 4.1 and Figure 4.2; bottom panel).

4.3 Discussion

We have shown that nuclear export of LXRα and LXRβ are energy-dependent but neither are Crm-1 dependent. In addition to these findings, we found that calreticulin plays a role in the regulation of nuclear localization of LXRs in a ligand-dependent manner. These findings indicate that LXRs are exported from the nucleus in the presence of LMB and the absence of RRL. Together these data indicate that LXRs are exported in the presence of ggPP and calreticulin but their localization is unchanged due to LXRs being re-imported in the nucleus (Prufer and Jackson unpublished data).
Figure 4.1 Nuclear export of LXRα requires energy but not Crm-1
Digitonin permeabilization experiments were performed in the presence of ggPP or LMB at 37°C or 4°C. HEK293 cells expressing YFP-LXRα were incubated for 1 hour in the presence of geranyl geranyl pyrophosphate (ggPP; 1µM) and/or leptomycin B (LMB; 2nM) as indicated after permeabilization with 50 µg/mL digitonin for 10 minutes on ice (permeabilized) or unpermeabilized.
Figure 4.2 Nuclear export of LXRβ requires energy but not Crm-1
Digitonin permeabilization experiments were performed in the presence of ggPP or LMB at 37°C or 4°C. HEK293 cells expressing YFP-LXRβ were incubated for 1 hour in the presence of geranyl geranyl pyrophosphate (ggPP; 1µM) and/or leptomycin B (LMB; 2nM) as indicated after permeabilization with 50µg/mL digitonin for 10 minutes on ice (permeabilized) or unpermeabilized.
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Emily Ann Jackson was born in New Orleans, Louisiana, to Brenda W. Jackson. Emily grew up in New Orleans, Louisiana, where she attended Our Lady of Lourdes Catholic School. She graduated from Eleanor McMain Magnet Secondary High School in May 2000. Emily began her undergraduate career in the fall of 2000 at Louisiana State University, where she majored in biological sciences. In the summer of 2004, Emily graduated with her Bachelor of Science from Louisiana State University. She entered the graduate program at Louisiana State University in the Department of Biological Sciences in the fall of 2005 as a doctoral student. Emily will graduate with her Master of Science degree in biochemistry on May 21, 2010.