2003

Isolation and Characterization of Estrogen Receptor- Interacting Proteins

Yun Kyoung Kang

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A thesis presented to the faculty of The Rockefeller University in partial fulfillment of the requirement for the degree of Doctor of Philosophy

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Dedication

To my family

Acknowledgements

I would like to appreciate Dr. Robert G. Roeder for giving me the opportunity to study such an exciting and challenging project in this stimulating environment. I would like to express my deep respect for his incredible passion and devotion for science and critical scientific mind, which has always and will have always inspired and influenced me.

I owe my deepest gratitude to my whole family. They are the solid basis for my integrity. In particular, I am very grateful to Hwa Jin Baek who has always supported me not only as a caring husband but also as a helpful scientific collaborator and my lovely daughter, Michelle Baek who has been the resource of happiness and peace. I am most indebted to my parents for their endless love, trust and sacrifice for all the years. I also thank my sisters and my brother for their love.

Many current and past colleagues in the Roeder lab have helped me with advice, materials and friendship. I am especially thankful to Dr. Sohail Malik for his kind advice, generous reagents, critical reading for this manuscript and friendship. I also thank Drs. Dong Kun Lee, Unkyu Kim, and Woojin An for their warm caring. I also appreciate the collaboration with Drs. Mohamed Guermah, Chaoxing Yuan, Wenzhu Zhang, Brian Chait, Vikas Palhan, Annika Wallberg, and Woojin An. I also sincerely acknowlege Drs. Jong Bok Yoon and Yuriko Suzuki for their freinship at the beginning of my graduate period. I thank Bala for her friendship and help. Much appreciation go to Wei Chen, Yali Dou, Kai Ge, Yan Luo, Claudia Mertens, Xiodi Ren, Soichiro Yamamura, Xin Yu, Jinsong Zhang,Xiaoting Zhang, Lei Zheng, Ernest Martinez, Tomas Oelgelschlager, Sean Stevens, Yong Tao, Zhengxin Zhang, Etsuko Uno, Armin Gamper, Jae Hoon Kim, Rachael Siegel, Jack Fu and Weizhen Wu. I would like to express much gratitude to Carmen-Gloria Balmaceda, Melissa Tellinghuisen and Helen Lynos for helping me with many lab-related business.

Finally, I would like to show my deep appreciation to my committee members, Drs. James Darnell, Bruce McEwen and for their advice, time and effort.

List of Abbreviations

ER	Estrogen receptor
LBD	Ligand binding domain
E_2	17β-estradiol
TRAP	Thyroid hormone receptor associated protein
ERKO	Estrogen receptor knockout
αERKO	ERα knockout
PR	Progesterone receptor
PRL	Prolactin
βERKO	ERβ knockout
DERKO	Double ERKO
DES	Diethylstilbestrol
TAM	Tamoxifen
RAL	Raloxifene
GEN	Genistein
HAT	Histone acetyltransferase
PMSF	Phenylmethylsulfonyl fluoride
DTT	Dithiothreitol
GST	Glutathione-S-transferase
MEF	Mouse embryo fibroblast
NP-40	Nonidet P-40
DMEM	Dulbeco's modified Eagle's Medium
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
SERMs	Selective Estrogen Receptor Modulators
ERE	Estrogen response element
DBD	DNA binding domain
AP-1	Activating protein-1
RAR	Retinoic acid receptor
IL-6	Interleukin 6
MCP-1	Macrophage chemoattractant protein
NF-κB	Nuclear factor B
C/EBP	CCAAT/enhancer binding protein
EGF	Epidermal growth factor
IGF-1	Insulin-like growth factor I
TGF	Transforming growth factor
ERAP	ER-associated protein
bHLH	Basic helix-loop-helix
PAS	Per/Arnt/Sim
RID	Receptor interacting domain
SRA	Steroid receptor RNA activator
HMT	Histone methyl transferase
CARM1	Coactivator-associated arginine methyltransferase 1
PRMT1	Protein arginine methyltransferase1
SMRT	Silencing mediator of RAR and TR

NcoR	Nuclear receptor corepressor
HDACs	Histone deacetylases
SHARP	SMRT/HDAC1 associated repressor protein
REA	Repressor of ER activity
SRB	Suppressor of RNA polymerase B
CTD	Carboxyl terminal domain
SMCC	SRB- and MED-containing Coactivator Complex
DRIP	Vitamin D receptor-interacting proteins
NAT	Negative regulator of activated transcription
ARC	Activator-recruited complex
CRSP	Coactivator required for Sp1
EM	Electron microscopy
PPARγ 2	Peroxisome proliferator-activated receptor γ2
HNF4	Hepatocyte nuclear factor 4
MAPK	MAP kinase
SUR	Suppressor of ras
hERβ	Human ERβ
f:Nut2	FLAG(f)-tagged Nut2 proteins
PBS	Phosphate-buffered saline
f:TR	FLAG(f)-tagged Thyroid hormone receptor
WT	Wild type
220KO	$TRAP220^{-/-}$
TR	Thyroid hormone receptor
ΔTRAP/MED	TRAP/Mediator-depleted nuclear extract

Abstract

Estrogen fulfills a variety of physiological roles through its nuclear receptors, estrogen receptors (ERs). The action of ERs is modulated and mediated by diverse interacting cofactors. The initial aim of this thesis work was to identify ligand-, subtype- and/or cell type-specific ER-interacting proteins.

I identified a number of nuclear extract-derived proteins that interact with immobilized ER ligand binding domains (LBDs) in a 17β-estradiol (E_2)-dependent manner. The most prominent of these are components of the thyroid hormone receptor associated protein (TRAP)/Mediator coactivator complex, which interacts with ERα and ERβ both in unfractionated nuclear extracts and in purified form equally efficiently. Although some of other interacting proteins displayed subtype specificity, the main focus of the work was to characterize the function of TRAP/Mediator in ER-dependent transcription for both ERα and ERβ.

Studies with extracts from TRAP220-/- mouse embryo fibroblasts (MEFs) revealed that the interactions between TRAP and ERs are dependent on TRAP220, a TRAP/Mediator subunit previously shown to interact with ER and other nuclear receptors in a ligand-dependent manner. The

physiological relevance of the in vitro interaction was further documented by the isolation of an ERα-TRAP/Mediator complex from HeLa cells expressing an epitope-tagged ERα lacking AB domain (f:ERαΔAB). Most importantly, the complete TRAP/Mediator complex was shown to directly enhance ER function in a highly purified cell free transcription system.

I further examined the roles for TRAP220 NR box in ER-TRAP/Mediator interaction. Both NR boxes were found to be important for ER-TRAP/Mediator interaction. Transient transfection assays with TRAP220-/- MEFs revealed that the absence of TRAP220 or mutations in either or both NR boxes attenuated modestly ER dependent transcription in TRAP220-/- MEFs. This indicated that TRAP220 and its NR boxes are required for the optimal transcrictipationl activity of ER. However, the observation of the significant E_2 responses in TRAP220-/- MEFs suggested the existence of alternative pathways for E_2 responses different from TRA/Mediator or the absence of natural constraints that impose a requirement for TRAP220 to overcome. Similarly, cell-free transcription assays also showed modest defect in function of TRAP/Mediator for ER dependent transcription when TRAP/Mediator contains TRAP220 NR mutants.

Chapter 1

Introduction

1.1. Estrogen Receptor

The transduction of extracellular signals into intracellular responses is a vital and fundamental process of all living organisms. It is the means by which communication between the genetic attributes of an organism and surrounding nature takes place. Through this system, the living organism can achieve active adaptation to their surroundings resulting in long-term evolutionary process. One major system of communication that has evolved in higher eukaryotes is the endocrine system, which is coordinated by chemical messengers called hormones. Hormones influence the actions of many tissues and thus affect many processes including metabolism, growth, development, emotion, and behavior, in addition to contributing to homeostasis (Goodman, 1996a). The steroid hormone, estrogen, is considered a female sex steroid hormone because it is produced mainly from the ovary and controls the growth, differentiation, and function of female reproductive and accessory sex tissues (Goodman, 1996b). However, estrogen also influences male reproductive and accessory sex tissues (Luconi et al., 2002). In addition to the reproductive tissues, non-reproductive tissues are also targets of estrogen. These

include skin, hair, bone, liver, pituitary, hypothalamus, cardiovascular system and behavioral centers in the brain (Farhat et al., 1996; Oursler, 1998; Toran-Allerand et al., 1999; Shupnik, 2002; McEwen, 2002).

How does estrogen affect target tissues? Cells appear to have evolved two ways to respond to estrogen signals. These are termed the genomic and nongenomic responses. First, the classical genomic action of estrogen is mediated through nuclear receptors called estrogen receptors (ERs) of the target cells by regulating the expression of specific subsets of genes that lead to long-term physiological effects. On the other hand, the nongenomic action of estrogen involves mechanisms that are not necessarily mediated through changes in gene expression. Instead, they are thought to be mediated through interactions between estrogen and cellular membrane components such as lipids and/or possible membrane receptors (McEwen and Alves, 1999; Watson and Gametchu, 1999). There are reports suggesting the existence of novel membrane receptors that are distinct from ERs and their involvement in these effects (Luconi et al., 2001; Nadal et al., 2000). However, a number of studies have provided evidence that a subpopulation of ERs are present in the cellular membrane

and play important roles in these rapid responses through intracellular signaling (Levin, 2002; Razandi et al., 1999). This has suggested that ERs are the primary mediator of estrogen actions in both the genomic and the nongenomic pathways.

1.1.1. ER is a member of nuclear hormone receptor family.

Two subtypes of ER have been identified so far: the classical ERα (Green et al., 1986; Greene et al., 1986) and the recently discovered ERβ (Kuiper et al., 1996). ERs are members of a large superfamily of nuclear receptors that function as ligand-regulated transcription activators. Like other nuclear receptors, ERs are modular proteins consisting of an amino-terminal region (A/B domain); a central DNA binding region (C domain); the carboxyl-terminal region (E domain) which is responsible for ligand-binding; the hinge region (domain D), which is located between the DNA and the ligand-binding domains; and the F domain, which is located at the extreme carboxyl terminus of the protein (reviewed in Gronemeyer, 1991).

ERs contain two transcriptional activation functions (AFs), which are responsible for the expression of target genes; the ligand-independent, amino-terminal AF-1 and

the ligand-inducible, carboxyl-terminal AF-2 (Tora et al., 1989). Both AF-1 and AF-2 are required to achieve maximal transcriptional activity of ER (Tzukerman et al., 1994) through the cooperative action (Chen et al., 2000; Kobayashi et al., 2000) but can also function independently with certain cell type and promoter specificities (Tzukerman et al., 1994). In particular, AF-1 activity exhibits cell type and promoter context specificities (Berry et al., 1990; Tora et al., 1989), displays different potency in each ER subtype (Hall and McDonnell, 1999; McInerney et al., 1998) and is regulated via phosphorylation cascades (Kato et al., 1995) by facilitating recruitment of cofactors (Tremblay et al., 1999). To date, a wide array of factors have been shown to interact with and enhance transcriptional activities of ERs. A large subset of these factors interact directly with the ER-LBD in a ligand and AF-2 dependent manner. As shown in three dimensional structures, upon ligand binding receptor undergoes conformational changes in the orientation of helix H12 within core AF-2 in order to allow cofactors to interact (reviewed in Pike et al., 2000).

1.1.2. Physiological roles of ER

The recent development of estrogen receptor knockout (ERKO) mice has provided suitable models to study the physiological roles of ERs (Couse and Korach, 1999; Mueller and Korach, 2001; Nilsson and Gustafsson, 2002) As expected, the phenotypes exhibited in ERα knockout (referred as αERKO) mice due to estrogen insensitivity have demonstrated significant roles of ERα in the reproductive system and accessory sex tissues (Couse and Korach, 1999). These include functions: (1) in proliferation and differentiation of cells that are critical to the function of the adult female reproductive tract and mammary gland; (2) as an essential component in growth factor signaling in the uterus and mammary gland; (3) in negative regulation of gonadotropin gene transcription and LH levels in the hypothalamic-pituitary axis; (4) as a positive regulator of progesterone receptor (PR) expression in several tissues; (5) in the positive regulation of prolactin (PRL) synthesis and secretion from the pituitary; (6) as a promotional factor in oncogene-induced mammary neoplasia; and (7) as a crucial component in the differentiation and activation of several behaviors in both female and male (Couse and Korach, 1999). Genetic ablation of ERα also revealed essential roles for ERα in certain aspects of male

reproduction, as reflected in phenotypes displayed in αERKO male mice such as production of abnormal sperm and the loss of intromission and ejaculatory response (Couse and Korach, 1999). Moreover, the phenotypes observed in αERKO mice have indicated more diverse roles of ERα in various nonreproductive tissues such as bone, brain and cardiovascular system (Mueller and Korach, 2001; Nilsson and Gustafsson, 2002). These aberrant phenotypes included: (1) growth arrest of longitudinal bones in both sexes as well as lower bone density in male mice, (2) increased aggression and infanticide in female mice as well as reduced aggression in male mice, (3) reduction of estradiol-induced angiogenesis and increase in serum apolipoprotein E, lower basal levels of vascular nitric oxide, increase in calcium channels and delayed cardiac depolarization (Couse and Korach, 1999). However, certain estrogen pathways in the αERKO female appear intact or unaffected, such as the ability of the uterus to successfully exhibit a progesterone-induced decidualization response, and the possible maintenance of an LH surge system in the hypothalamus including the proliferative and differentiative actions critical to the function of the adult female reproductive tract and mammary gland (Couse and Korach, 1999).

Compared to αERKO mice, ERβ knockout (βERKO) mice showed mostly normal phenotypes in both female and male mice, except for subfertility in female mice that may be due to infrequent and inefficient ovulation (Krege et al., 1998). This suggested that ERβ is essential for normal ovulation efficiency but not for female or male sexual differentiation, fertility, lactation or certain other nonreproductive system (Krege et al., 1998). Loss of both receptors in the double ERKO (DERKO) mice leads to a striking sex reversal phenotype characterized by postnatal loss of oocytes and redifferentiation of the remaining somatic cells to Sertoli-like cells (Couse et al., 1999). This surprising ovarian phenotype of DERKO mice is distinct from that of the individual ERKO mice (Couse et al., 1999). This indicates cooperative actions of both receptors that are required for the maintenance of germ and somatic cells in the postnatal ovary (Couse et al., 1999). Analyses of the phenotypes of αERKO, βERKO and DERKO mice suggested specific contribution of each subtype of ERs as well as cooperative actions of both ERs in estrogen signaling (Couse et al., 1999).

1.1.3. Actions of ER

What is the molecular basis of the above-mentioned physiological effects? What is the molecular mechanism of ER action that mediates estrogen signaling? This has been one of the most intensively studied subjects due to its clinical and pharmaceutical relevance. Long lasting clinical needs for novel hormone replacement therapies that retain the beneficial effects of estrogen, and at the same time do not possess the higher risk of breast cancer has driven the pharmaceutical industry to develop new Selective Estrogen Receptor Modulators (SERMs) that modulate ER in a tissue-selective manner. With respect to this, it is very important to understand the molecular mechanism of ER action underlying the behavior of SERMs.

1.1.3.1. Genomic actions of ER

The genomic actions of ERs have been well established. As ligand-modulated, DNA-binding transcription factors, ERs regulate expression of their target genes upon ligand binding. The process of genomic action of ER, thus, can be dissected into four major components, which together contribute to the modulation of ER function: ligands, DNA, ER and cellular factors.

1) Ligands

Todate thousands of ligands for ERs have been synthesized. These compounds can be classified into agonist, partial agonist (partial antagonist) and pure antagonist depending on the ER response to these ligands.

Early studies using protease digestion assays suggested that ligand binding induced a conformational rearrangement in the ligand binding domain (LBD) and hinted that the structure of ER-ligand complex might contribute to its functional specificity (McDonnell et al., 1995). Recent crystallographic analyses confirmed this idea by showing that different functional classes of ligands induce distinct conformations of ER in the orientation of helix H12 (reviewed in Pike et al., 2000). Agonist and antagonist bind at the same site within the core of the LBD but demonstrate different binding modes (Brzozowski et al., 1997; Pike et al., 1999; Shiau et al., 1998). In the case of agonists such as estradiol (E_2) and diethylstilbestrol (DES), the ligand is completely encased within the ligand-binding pocket and helix H12 is aligned over the pocket (Brzozowski et al., 1997; Shiau et al., 1998). This conformation allows peptides containing the short signature sequence motif (LXXLL motif where L is Leucine and X is any amino acid) known as the NR box to interact with the static region of

the coactivator recognition groove in AF-2 domain (Brzozowski et al., 1997; Shiau et al., 1998). The binding of the partial antagonists, tamoxifen (TAM) and raloxifene (RAL), however, is accompanied by major structural reorganization in the ternary structure in both ERs (Brzozowski et al., 1997; Pike et al., 1999; Shiau et al., 1998). The bulky side chain of the ligands protrudes out of the ligand-binding pocket. The resulting steric clashes (1) inhibit helix H12 from covering the ligand-binding pocket and instead, (2) force helix H12 to extend to the coactivator recognition groove, thereby imitating the interactions of ER with the NR box through its NR-box like sequence (LXXML where L is Leucine, X is any amino acid, and M is Methionine) (Shiau et al., 1998). The structure of the ERβ LBD bound to the ERβ partial agonist, genistein (GEN), reveals that ligand binding can stabilize yet another conformation of H12 (Pike et al., 1999). In this complex, H12 is bound over the ligand-binding pocket in a position such that it occludes the coactivator recognition groove partially (Pike et al., 1999). However, the functional significance of this conformation of the LBD is unclear (Pike et al., 1999). The side chain of ICI 164,384, a pure antagonist, binds directly to the coactivator binding cleft of ERβ,

causing physical blockade of H12 alignment (Pike et al., 2001). Complete destabilization of H12 configuration and concomitant exposure of a large hydrophobic patch on the surface of ER-LBD is thought to be an account for rapid degradation of ICI-bound ER and such degradation is considered as a major mechanism of pure antagonism (Pike et al., 2001).

2) DNA

As a template, DNA provides the genetic information for transcription. DNA also supplies its own regulatory information through gene-specific combinatorial arrangement of enhancer elements recruiting cognate sequence-specific DNA-binding transcription activators and distinct architecture of the core promoter. In this way, DNA is the key component to impose unique specificity to expression of a given gene. Consistent with this notion, DNA participates in important aspect(s) of regulation of ER function.

Comparison of the promoter sequences of estrogen-responsive genes led to the identification of the consensus estrogen response element (ERE), a palindrome of PuGGTCA motifs separated by 3 bp (Evans, 1988; Green and Chambon, 1988). ER interacts with DNA as dimers, with

one receptor interacting with each sequence motif (Gronemeyer and Meyer, 1991). The sequence-specific recognition of these elements is through the DNA binding domain (DBD) region of ER, which consists of two Cys4 zinc fingers (Gronemeyer and Meyer, 1991). Mutation and crystallographic analyses revealed the molecular determinants dictating the sequence specificity of interactions between ERs and specific DNA elements (Danielsen et al., 1989; Mader et al., 1989; Umesono and Evans, 1989).

The composition of the consensus sequence and the spatial organization of the motifs together with flanking sequences are important for the affinity and the specificity of ER binding. Although a few promoters of estrogen target genes contain EREs that match the consensus sequence (Xenopus Vitellogenin A2, human EFP, human EBAG9) (Ikeda et al., 2000; Inoue et al., 1993; Klein-Hitpass et al., 1986), most elements identified to date are not perfectly palindromic (reviewed in Klinge, 2001). Deviations from the consensus in one half-site reduce ER-binding affinity in vitro (Nardulli et al., 1996). It is interesting to note that the pS2 and the cathepsin D promoters, which both contain a T to C mutation in the PuGGTCA motif, were reported to function

poorly in endometrial carcinoma Ishikawa cells, but efficiently in breast carcinoma MCF7 cells (Miralles et al., 1994). Moreover, it has been suggested that the individual ERE sequences induce specific conformational changes in ER (Wood et al., 2001 and references therein). This ERE-induced allosteric modulation of ER conformation has been shown to affect the recruitment of specific coactivator proteins leading to differential gene expression from target genes containing divergent ERE sequences (Hall et al., 2002; Wood et al., 2001). These studies proposed that diversity of ERE sequences would provide mechanisms to determine the specificity of target gene activation by ERs.

3) ER

The surprising discovery that ERβ is encoded by a gene different from ERα (Kuiper et al., 1996), has revealed the complexity of ER action and led to the reevaluation of the mechanism of ER function. ERβ shows an overlapping but nonidentical tissue distribution compared to ERα (Kuiper et al., 1997). ERβ is predominantly expressed in testis, prostate, ovary, liver, and specific regions of the brain whereas ERα has been detected in several mouse tissues including the

mammary gland, uterus, ovary, liver, kidney, heart and testis (Couse and Korach, 1999). Some tissues, such as the thyroid gland, epididymis, bone and brain, express both ERs (Couse and Korach, 1999). However, very few, if any, of these tissues express both ERs in the same cell types implying that these receptors have distinct functional roles in mammals. For example, although rat ovary contains both ERs, ERβ is located in the granulosa cells, whereas ERα is localized to the surrounding thecal cells (Hiroi et al., 1999; Sar and Welsch, 1999).

In primary structure, human ERβ displays some homology to ERα (reviewed in Pettersson and Gustafsson, 2001). The maximal homology between ERα and ERβ lies in their DBDs (96% identity) (Ogawa et al., 1998) indicating that both receptors share the same EREs (reviewed in Pettersson and Gustafsson, 2001). The LBD is also relatively conserved (53% identity) between these two receptors (Ogawa et al., 1998). Consistent with the primary structural homology, overall similarities in three dimensional structures of the LBD of both receptors were demonstrated in crystallographic studies (Pike et al., 1999; Shiau et al., 2002). Although ERα and ERβ exhibited similar binding affinities for most ligands (Kuiper et al., 1997), several new compounds have been

shown to bind preferentially to specific ER subtypes (Barkhem et al., 1998; Kuiper et al., 1998). This observation has led to the recent development of ERβ selective ligands (Katzenellenbogen et al., 2000; Sun et al., 1999). The N-terminal domains, namely the A/B domains of ERα and ERβ, share the least homology (30% identity)(Ogawa et al., 1998). Considering the observation that the A/B domain encompasses AF-1, whose activity acts in cell type- and promoter context-specific fashions (Berry et al., 1990), the poor homology between A/B domains of ERα and ERβ implies that tissue-selective actions of SERMs could be partly but significantly due to functional distinction between two subtypes of ERs that has been attributed to the structural differences between the A/B domains. Consistent with this idea, ERα contains a strong AF-1 activity that results in the partial agonism of some antiestrogens such as TAM and RAL. In contrast, ERβ shows, under the same conditions, negligible AF-1 activity and results in pure antagonism by TAM and RAL (McInerney et al., 1998) . Moreover, ERα and ERβ shows opposite responses to the ligands on AP-1 promoters (Paech et al., 1997). In the presence of estradiol, ERα activates transcription whereas ERβ represses transcription, but, in the presence of

antiestrogens, ERβ is ten times more potent than ERα as a transcription activator (Paech et al., 1997). This difference also has been suggested to be a result of the structural difference between the A/B domains of ERα and ERβ (Webb et al., 1999). The studies reporting the existence of subtype-specific cofactors (Endoh et al., 1999; Watanabe et al., 2001; Wu et al., 2001) provided evidence that these factors interact with ERα AF-1 and mediate its activity. Taken together, one can speculate that the different structural features the A/B domains of ERα and ERβ contribute to the functional distinction between ERα and ERβ through recruitment of different subset of cofactors.

4) Cellular factors

Lastly, the factors in the cellular environment contribute significantly to the regulation of ER function. As transcription factors, ERs communicate with a wide variety of factors such as gene-specific components of enhanceosomes, cofactors, chromatin remodeling factors, and general transcription factors (GTFs) (Beato and Sanchez-Pacheco, 1996). ERs also communicate with the components of other signal transduction pathways (Smith, 1998).

It is well established that the cellular milieu changes during the processes of proliferation, differentiation and/or development. At a given time point in a certain stage of differentiation and development, each cell has specific composition of factors depending on its tissue context. There are many types of tissue- and/or stage-specific transcription factors that regulate cell function (Karin et al., 1990; Maniatis et al., 1987; Struhl, 1991). Additionally, the existence of tissue-specific transcription cofactors has also been discovered (Luo et al., 1992; Luo and Roeder, 1995). At least one component of general transcription machinery also displays tissue-dependent variation (Hansen et al., 1997). Thus, the composition of available factors differs considerably depending on the cell type and stage of differentiation. Even in the same cell, each gene obtains its specificity in transcriptional regulation through unique combinatorial arrangements of the enhancer elements, in addition to the individual architecture of the core promoter. Therefore, ER-mediated transcriptional activation acquires cell-and/or promoter specificity via such specific cellular milieu and gene context.

4-1) Transcription factors

It has been shown that ER functions indirectly through protein-protein interactions with other DNA-binding transcription factors. The most studied example is the ER and activating protein-1 (AP-1) interaction. Ligand-activated ERα can interact with, and positively regulate, the collagenase gene promoter by associating with the AP-1 transcription factor complex within target cells (Webb et al., 1995). In this particular system, SERMs, such as TAM, manifest agonist activity. The ER-AP-1 activity is particularly strong in endometrium-derived cell lines, where TAM is an agonist, whereas it is less active in cultured breast cancer cells, where TAM has antagonist activity (Webb et al., 1995). This study hints at a correlation between the ability to activate the ER–AP-1 complex and the ability of a cell to support the partial agonist activity of TAM. Moreover, the observation of the opposite responses of each subtype of ERs to E_2 versus TAM on AP-1 promoters (Paech et al., 1997) further corroborates the idea of the involvement of ER-AP-1 complex in tissue specific action of SERMs.

Another well-known example is cooperative action of ER and Sp1. In addition to the above-described ERE sites, GC boxes (GGGGCGGGG) or GT/CACCC boxes (GGTGTGGGG), which are binding sites for the transcription factor SP1, were

found to be associated with individual PuGGTCA motifs in regulatory regions of an increasingly large number of estrogen-responsive target genes (reviewed in Safe, 2001). The importance of both the half-ERE and the Sp1 site for estrogen response have been indicated by studies using reporter plasmids containing the cathepsin D promoter or the RARα promoter. It was shown that mutations in either the Sp1 or the half-ERE part of composite motifs were no longer inducible by estrogen (Krishnan et al., 1994; Rishi et al., 1995). Cooperativity between ER and SP1 was also observed with promoters containing palindromic EREs, such as that found in the vitellogenin A1 and the rabbit uteroglobin promoter (Batistuzzo de Medeiros et al., 1997; Scholz et al., 1998). However, in some cases ERs may function cooperatively with Sp1 activity even without ERE. Deletion of the PuGGTCA motif in the promoter of the hsp27 gene did not affect its responsiveness to estrogen (Porter et al., 1997). Moreover, Sp1 sites in the promoters of the c-fos, bcl-2 and IGFBP4 genes were found to be sufficient for induction by estrogen (Safe, 2001). Interestingly, the effects of estrogen on Sp1 activity exhibited specificity depending on the cell line and the type of ER expressed. HeLa cells showed no activation of

Sp1 by estrogen when ERα was expressed, and a slight repression with ERβ (Saville et al., 2000). On the other hand, Sp1 sites were sufficient for stimulation by ERα but not ERβ in MCF7 and MDA-MB-231 breast cancer cells (Saville et al., 2000). These studies suggested that ability to support cooperative actions of ER-Sp1 would be another possible way of cell type-specific actions of ER.

There are other systems where these indirect transcriptional regulatory pathways have been shown to be important. For example, it has been shown in rodents that the retinoic acid receptor (RAR) promoter is upregulated both by estrogens and antiestrogens (Elgort et al., 1996). Remarkably, even the pure antiestrogen ICI 182,780 functions as an agonist on this promoter (Elgort et al., 1996). Mutational analysis of the RAR promoter revealed that, in this system, ERα exerts its regulatory activities in an indirect manner through a pre-bound transcription factor (Elgort et al., 1996). The identity of this factor remains to be determined (Elgort et al., 1996).

In a different system, antagonist-bound ER stimulated quinone reductase gene transcription through a previously defined electrophile-response element (Montano and Katzenellenbogen, 1997). In this system, ERβ is a

more potent activator than ERα through stronger interactions with the human homologue of *Xenopus* gene which prevents mitotic catastrophe (hPMC2) (Montano et al., 1998; Montano et al., 2000). These findings indicated that ERs can contact the transcription apparatus in an indirect manner through several distinct types of protein–protein interactions meaning that there are at least two distinct mechanisms by which the ER can function as a transcriptional activator in target cells.

There is some evidence, however, that ER, in some instances, can also function as a transcriptional repressor through these indirect regulatory pathways. In bone, for example, it has been shown that estrogens and antiestrogens effectively suppress production of interleukin 6 (IL-6), a cytokine required for osteoclastogenesis and osteoblastogenesis (Girasole et al., 1992; Jilka et al., 1992; Ray et al., 1994). The repression of IL-6 expression by ERs in osteoblasts and bone marrow stromal cells may explain in part the protective effects of circulating estrogen on bone density. Upon activation by ligand, the ER physically interacts with the p65 subunit of nuclear factor B (NF-κB), thus blocking its ability to bind to target sequences located within the regulatory regions of the

IL-6 genes (Stein and Yang, 1995). In this process, only regions C to F of ERs are required for NF-κB repression suggesting that it does not involve ER binding to DNA. This regulatory paradigm might extend beyond NF-κB, as it has been shown that the ER can inhibit the transcriptional activity of CCAAT/enhancer binding protein (C/EBP) and GATA-1 in a similar way (Blobel et al., 1995; Stein and Yang, 1995). All together, these findings strongly support the physiological relevance of the inhibitory activity of ER.

4-2) Cross-talk with signal transduction pathways

Independent of estrogen signaling, other signal transduction pathways also regulate the transcriptional activity of ERs. Treatments with growth factors such as epidermal growth factor (EGF), insulin, insulin-like growth factor I (IGF-1), and transforming growth factor (TGF)-β can modulate transcriptional activity of ER through phosphorylation in the absence of estrogen (Smith, 1998). Early studies showed that EGF could mimic effects of estrogen in the mouse reproductive tracts and pretreatment of mice with the pure anti-estrogen greatly diminished the uterine response to EGF (Ignar-Trowbridge et al., 1992) suggesting the ER mediates this effect. The

lack of uterine response to EGFs in αERKO mice as described above (Couse and Korach, 1999) further supported the physiological relevance of EGF signal transdunction pathway in function of ERα. Typically, such cross-talk involves the direct phosphorylation of specific serine residues in AF-1 domains of both ERs by MAP Kinase (Kato et al., 1995; Tremblay et al., 1999). The net effect of these phosphorylation events is to potentiate AF-1 activity by facilitating recruitment of coactivators to the A/B domain in a ligand-and AF-2-independent manner. In the case of ERα, the phosphorylation on Ser-118 of ERα by MAPK facilitates the recruitment of another cofactor, p68 (Endoh et al., 1999). Additionally, the phosphorylation of ERβ by MAPK resulted in a direct interaction between AF-1 and SRC-1, independent of ligand and the entire AF-2 sequences (Tremblay et al., 1999). Such phosphorylation cascades has been shown to be involved in the TAM-mediated agonism through modulation of AF-1 or its cofactors (Feng et al., 2001).

The observation of E_2-mediated downregulation and TAM-induced upregulation of HER-2/neu (a member of the EGFR family) synthesis (Newman et al., 2000) suggested

the existence of a cross-regulatory loop between estrogen signaling and other signal transduction pathways.

4-3) Cofactors

It is clear from the above-described studies that the regulatory mechanisms of ER function are diverse and complex. However, it appears that the regulatory information provided by distinct ligands, diverse EREs, different attributes of ER subtypes, differential cellular ability to support cooperative action with other transcription factors or signal transduction pathways are integrated to contribute to the recruitment of cofactors. This is the hypothesis from which my thesis work has started (see below). With this view, the dominant effect of ligands, agonism versus antagonism, depends on the potential of the ligand to either induce the correct conformation or disrupt the active conformation or, in other words, to respectively stimulate or prevent the correct binding of coactivators.

In support of this idea, a recent report (Shang and Brown, 2002) provided compelling evidence that the differential recruitment of cofactors to nonclassical promoters (such as c-Myc) and/or modulation in cofactor expression (such as SRC-1) is the primary determinant for

agonism of TAM in either breast or endometrial cancer cells. This indicated that cell-type and promoter-specific differences in cofactor expression and/or recruitment determine the cellular response to SERMs.

1.1.3.2. Nongenomic actions of ER

Estrogen can modulate protein function via nongenomic actions that are very rapid and cannot be accounted for by changes in transcription. Increasing number of studies have indicated that a subpopulation of ERs located in the cellular membrane play important roles in these rapid responses through intracellular signaling (Levin, 2002; Pappas et al., 1995; Razandi et al., 1999). For example, ERα has been shown to regulate MAP kinase activity in MCF-7 breast cancer cells through interaction with the SH2 domain of Src (Migliaccio et al., 1996) and lead to induction of cell proliferation (Castoria et al., 1999). ERβ acts by a similar mechanism in LNCaP prostate cancer cells (Migliaccio et al., 2000). Modulation of MAP kinase activity by estrogen has also been described in bone cells. Kousteni et al demonstrated that the estrogen-induced antiapoptotic effect is mediated by activation of Src/Shc/ERK signaling pathway via ER action

(Kousteni et al., 2001). Importantly, this study first provided evidence of the nongenomic effect of ER dissociated from transcriptional activity of ER by using synthetic ligands and peptide antagonists (Kousteni et al., 2001). Estrogen also regulates neuronal excitability (McEwen and Alves, 1999), intracellular calcium concentration, cyclic AMP synthesis and phosphoinositide turnover (reviewed in Kelly and Levin, 2001). Recently, ERα was shown to interact with the p85α regulatory subunit of the phosphatidylinositol-3-OH (PI3) kinase, resulting in increased enzymatic activity in vascular endothelial cells and leading to the activation of protein kinase B/Akt as well as endothelial nitric oxide synthase and the release of nitric oxide (Simoncini et al., 2000). This regulatory pathway may mediate the cardiovascular protective effects of estrogens, and appears to be specific to ERα and not ERβ (Hisamoto et al., 2001).

These mechanisms of estrogen action also contribute to gene expression via indirect pathways leading additional complexity in the regulation. It has been suggested that regulation of immediate early genes egr-1 and c-fos by estrogen in MCF7 cells is mediated by their serum response elements via non-genomic activation of the

raf-MAPK signaling pathway (Duan et al., 2001; Pratt et al., 1998). The function of numerous transcription factors including AP-1 and ERs themselves is regulated by phosphorylation (Kato et al., 1995; Whitmarsh and Davis, 1996). Therefore, the modulation of kinase activities by ERs is likely to contribute to fine-tuning of transcriptional regulation depending on the combination of transcription factors bound to a given promoter. In this way, non-genomic and genomic actions of estrogen may be intimately linked via cross-regulatory loops.

The complexity of action mechanisms of ERs suggests that nature has engineered multiplicity into ER biology to fulfill different functions in different cells.

1.2. ER-interacting proteins

The actions of ER result from the interplay of all of factors that are involved. As described above, ER-mediated transactivation is the process of the intercommunication between ER and the transcription machinery. Thus, the distinct characters of ligands and different attributes of ER subtypes are eventually integrated into the transcription machinery. Among the

main players in this process are the cofactors because they integrate differential signals and diversify the regulation to amplify specificity of transcription. A growing number of proteins have been identified in this category through its direct interaction with ER as well as other nuclear receptors in a ligand-dependent manner.

1.2.1. SRC/p160 family

ER-associated protein (ERAP)140 and ERAP160 (subsequently cloned as SRC-1 which is the first member of SRC/p160 family, see below) were identified through GST pull down assays as the first ER interacting proteins (Halachmi et al., 1994). The potential role of ERAP160 in ER functions was suggested by the observation that it failed to interact with either antagonist-bound ER or with transcriptionally defective mutants of ER (Halachmi et al., 1994). SRC-1 was originally cloned through its interaction with LBD of PR by yeast two-hybrid analysis (Onate et al., 1995). The second member of this family is identified as TIF2/GRIP1/NcoA-2/SRC-2 (Hong et al., 1997; Voegel et al., 1996). Concomitantly, AIB1/pCIP/ACTR/RAC3/TRAM-1/SRC-3 (Anzick et al., 1997; Chen et al., 1997; Li et al., 1997; Takeshita et al.,

1997; Torchia et al., 1997) was discovered as the third member. These proteins share a conserved domain structure including the most conserved N-terminal basic helix-loop-helix (bHLH) and Per/Arnt/Sim (PAS) domains that are implicated in protein-protein interactions (Aranda and Pascual, 2001). At the C-terminal end of the proteins reside two autonomous activation domains, a glutamine-rich domain, and regions important for interaction with CBP (Torchia et al., 1997) and for the weak intrinsic HAT activity (Spencer et al., 1997). In the central part are located receptor interacting domains (RIDs) containing three LXXLL motifs that are essential for the interactions with nuclear receptors (Heery et al., 1997). These motifs have been implicated in determining the specificity of the interaction with nuclear receptors through differential requirement of each LXXLL motif in the interaction with specific receptors (McInerney et al., 1998). For instance, ERα preferentially interacted with the second motif and only one motif was sufficient to support stable interactions. The flanking sequences also contributed to the specificity of interaction and are important for SRC-1 function as well as interaction with ERα (McInerney et al., 1998).

The functional significance of these proteins was well established by their ability to function as bona fide coactivators in vivo. For instance, SRC-1 showed a broad range of specificity in its function as a coactivator for several nuclear receptors as well as other transcription factors such as AP-1 and NF-κB. The important role of SRC-1 in ER functions was suggested by its stimulatory effect on the transcriptional activity of both ERα and ERβ and this effect was inhibited by antiestrogens (Onate et al., 1995; Tremblay et al., 1997). Moreover, the capability to reverse the ER-mediated squelching effect on PR transactivation indicated that this protein is a limiting factor that is commonly recruited by both ER and PR. SRC-1 is also implicated in cooperative synergism between AF-1 and AF-2 functions in ER through mediating functional interaction between AF-1 and AF-2 domains. Interestingly, TIF2 (a SRC-2) can stimulate ligand-dependent transcriptional activity only by ERα (Voegel et al., 1996) but not by ERβ (Bramlett et al., 2001). However, GRIP1, another SRC-2, was shown to function as a very strong coactivator for ERβ on the collagenase promoter (Webb et al., 1998). SRC-3 also showed functional preference in that it selectively enhanced the transcriptional activity of ERα

over that of ERβ although it can interact with both ERα and ERβ (Suen et al., 1998). These studies suggested that the specificity of ER functions is partly-this may be significant-dictated by the intrinsic functional features of SRC. This is consistent with the view that cofactors are major players in amplifying the specificity of ER function (reviewed in McKenna et al). Generation of knockout mice lacking SRCs has illuminated their physiological functions. The SRC-1 knockout mice were viable and fertile but exhibited growth reduction in estrogen target tissues (Xu et al., 1998). This included defects in uterine response to estradiol and mammary gland development in pregnant mice (Xu et al., 1998). Despite its broad functions as seen in cellular assays, the SRC-1 knockout mice showed relatively restricted defects. This, coupled with the observation of increase in TIF2 mRNA expression in SRC-1 knockout mice, indicates that certain compensatory mechanisms are involved in cofactor functions (Xu et al., 1998). Disruption of the SRC-3 gene in mice resulted in more dramatic phenotypes exhibiting growth retardation, delayed puberty, decreased reproductive function and blunted mammary gland development (Xu et al., 2000). Together with the original observation of the amplification of the SRC-3/AIB1 gene

in breast cancer (Anzick et al., 1997), the defects in the mammary gland development suggested a significant role of this cofactor in this tissue.

1.2.2. P300/CBP

P300 and CBP were originally isolated by their respective interaction with E1A (Eckner et al., 1994) and cAMP-regulated enhancer binding protein (CREB) (Kwok et al., 1994). They are now viewed as cointegrator proteins that function as common cofactors for diverse transcription factors to collate multiple signals into an integrated cellular response (Kamei et al., 1996). In this way, these proteins are involved in many important cellular processes including development, differentiation and oncogenesis (Goodman and Smolik, 2000). The involvement of p300/CBP in nuclear receptor functions has been suggested by gene deletion experiments, nuclear injection of blocking antibodies and transfection assays (Chakravarti et al., 1996; Chen and Okayama, 1987; Hanstein et al., 1996; Kamei et al., 1996; Kawasaki et al., 1998; Yao et al., 1996; Yao et al., 1998). Although direct interaction of p300/CBP with ERα through its RID has been clearly demonstrated in vivo and in vitro

(Hanstein et al., 1996), p300/CBP is now believed to be recruited to ERα via SRC-1 in the cell (Hanstein et al., 1996; Torchia et al., 1997; Yao et al., 1996). With its potent intrinsic HAT activity (Ogryzko et al., 1996) p300/CBP has been believed to play a central role in ER dependent transcription by remodeling chromatin. This has been clearly demonstrated by the study with the cell free transcription assays using chromatin template (Kraus and Kadonaga, 1998). Similar to SRC-1, p300/CBP has been also shown to mediate synergism between AF-1 and AF-2 (Kobayashi et al., 2000) through its interaction with AF-1 domain of ERα and by facilitating phosphorylation-dependent recruitment of SRC-1 to AF-1 domain of ERβ (Tremblay and Giguere, 2001).

1.2.3. AF-1 interacting proteins

Recently, MMS19, the human homologue of the yeast DNA repair enzyme has been found to interact with the AF-1 of ERα (Wu et al., 2001). Although it is not clear what the physiological relevance of this interaction is it might facilitate recruitment of RAC3 to the N-terminus of ERα through its interaction with PAS domain of SRC-3/RAC3 (Wu et al., 2001).

Surprisingly, an unusual RNA molecule called Steroid receptor RNA activator (SRA) has recently been described as an AF-1 specific cofactor for several steroid receptors including ERs (Lanz et al., 1999). SRA was found to be recruited to ERα via a subfamily of RNA-binding DEAD-box proteins called p68/p72 which were identified as ERα AF-1 specific coactivators (Endoh et al., 1999; Watanabe et al., 2001). These proteins interact directly with AF-1 region of ERα but not of ERβ. This interaction was facilitated by phosphorylation by MAPK and led to potentiation of AF-1 activity of ERα (Endoh et al., 1999; Watanabe et al., 2001).

1.2.4. Other ER-interacting proteins

There are many other factors that have been shown to interact with ERs and to stimulate ER dependent transcription including GTFs such as TBP (Sadovsky et al., 1995), TFIIB (Sabbah et al., 1998), and TFIIH (Chen et al., 2000b). Importantly, TFIIH has been shown to phosphorylate Ser-118 in a ligand-dependent manner through its interaction with ERα (Chen et al., 2000b). This study proposed the novel mechanism by which AF-1 activity can be regulated by ligand and AF-2 activity. Along with several HATs other chromatin remodeling

factors such as SWI2/SNF2 (Ichinose et al., 1997) and BRG-1 (DiRenzo et al., 2000) have been implicated in ER dependent transcription. These proteins interact directly with LBD of ERα in a ligand-dependent manner (DiRenzo et al., 2000; Ichinose et al., 1997). This interaction absolutely required AF-2 domain (DiRenzo et al., 2000; Ichinose et al., 1997). In particular, BRG-1 is required for the function of SRC-1 and p300/CBP suggesting their cooperativity for ER function (DiRenzo et al., 2000). Another emerging group of proteins that are involved in nuclear receptor function by chromatin remodeling is histone methyl transferase (HMT) (Kraus and Wong, 2002). Coactivator-associated arginine methyltransferase 1 (CARM1)(Chen et al., 2000a; Chen et al., 1999), protein arginine methyltransferase1 (PRMT1) (Koh et al., 2001) and PRMT2 (Qi et al., 2002) have been shown to enhance ER dependent transcription synergistically with SRC-1/p160 through their direct interaction with the AD2 activation domain of SRC-1/p160 but not directly with ER indicating their role as secondary factors. Another important factor for ER function is cyclin D1 indicating the tight link between ER function and the cell cycle. Cyclin D1 (without its CDK partner) was found to stimulate ER dependent transcription via direct interactions, which

were independent of ligand by recruiting SRC-1 (Zwijsen et al., 1998; Zwijsen et al., 1997). The physiological relevance was further highlighted by the recent demonstration that the interaction between ER and cyclin D1 was modulated by levels of cAMP in mammary epithelial cells (Lamb et al., 2000).

Other cofactors such as TR-binding protein (TRBP) (Ko et al., 2000), transcription intermediary factor 1 (TIF1) (Thenot et al., 1997), Coactivator independent of AF-2 function (CIA) (Sauve et al., 2001), E6-AP (Nawaz et al., 1999) and PPARgamma-coactivator-1 (PGC-1) (Tcherepanova et al., 2000) were found to enhance ER dependent transcription through their interaction with ERs. The physiological relevance of the interaction of these proteins with ERs remains to be studied.

1.2.5. Corepressors

Silencing mediator of RAR and TR (SMRT) (Horlein et al., 1995) and nuclear receptor corepressor (NcoR) (Chen and Evans, 1995) interact with the hinge region of RAR and TR in the absence of ligand and mediate basal repression of their respective target genes by recruiting many other factors such as histone deacetylases (HDACs) and Sin3 to form a repression complex (Jepsen and

Rosenfeld, 2002). This complex coordinates deacetylation of histones resulting in a condensation of chromatin and subsequent repression in transcription (Jepsen and Rosenfeld, 2002). Although the importance of these corepressors in nonsteroid hormone receptor function has been well known, their involvement in steroid receptor function was not yet fully demonstrated. However, ER has been found to interact with NcoR/SMRT and this interaction inhibits transcriptional activation in the presence of partial agonist such as TAM (Jackson et al., 1997; Smith et al., 1997). Furthermore, SRC-1 mediated TAM agonism was overcome by coexpression of SMRT indicating that these two proteins compete for access to partial agonist-bound receptor (Jepsen et al., 2000). This finding suggested that the expression level of these coregulators could be a determinant of tissue-selective agonism or antagonism of TAM (Jepsen et al., 2000). Further evidence for the involvement of NcoR/SMRT in ER function came from the discovery of SMRT/HDAC1 associated repressor protein (SHARP) by yeast two-hybrid analysis through the C-terminus of SMRT (Shi et al., 2001). This potent corepressor can attenuate SRA-induced ER transactivation by squelching SRA through the interaction of its RNA binding motifs with SRA and forming a complex

with several other corepressor proteins including HDACs (Shi et al., 2001). Interestingly, its mRNA level is induced in the breast cancer cell line MCF-7 upon treatment with E_2 suggesting possible participation of SHARP in the feedback mechanism for the attenuation of the hormonal response (Shi et al., 2001).

The effort to find a bona fide corepressor for ER has resulted in the cloning of REA (repressor of ER activity) (Delage-Mourroux et al., 2000). This protein represses the activity of both ERα and ERβ through interaction with ERs in the presence of antagonists but does not affect transcription by other nuclear receptors. REA-dependent repression could overcome SRC-1 mediated stimulation of ER through its LXXLL motif.

1.3. TRAP/Mediator

The necessity for precise control of specific gene expression during complicated cellular processes creates numerous regulatory layers on the transcription process. This results in a great number of components that are involved in transcription. However, these protein components of the transcription machinery can be grouped into four categories by their functions: (1) sequence-

specific DNA-binding transcription factors (Mitchell and Tjian, 1989); (2) chromatin remodeling factors including ATP-dependent remodeling factors and histone-modifying factors (Narlikar et al., 2002); (3) GTFs that are required for accurate transcription initiation from core promoters (Roeder, 1996); and (4) cofactors (Roeder, 1998). Cofactors, used loosely, include chromatin remodeling factors. However, in this section, cofactors are more specifically defined as the factors that are not essential for basal transcription but that are necessary for the optimal induction or repression by sequence-specific transcription factors without having intrinsic site-specific DNA binding activity and function beyond chromatin remodeling steps. The ultimate goal of the transcription activation can be represented as the facilitation of preinitiation complex (PIC) formation on the promoter (Keaveney and Struhl, 1998; Roeder, 1996). However, the access to the promoter is blocked by intrinsic structural features of chromatin. Therefore, the primary actions of transcription activators are (1) recruitment of a chromatin remodeling factors (Narlikar et al., 2002) and (2) induction of quantitative and qualitative changes of GTFs to promote PIC formation on the promoter (Roeder, 1996). Although many of

transcriptional activators can directly and physically interact with chromatin remodeling factors (Narlikar et al., 2002) and/or GTFs (Roeder, 1996), a large body of evidence suggested the major contribution of cofactors in effecting the communication among transcription activators, chromatin remodeling factors and/or GTFs through protein-protein interactions (Roeder, 1998) .

1.3.1. Discovery of Mediator

The concept of cofactors originally emerged from the "squelching effect" in which one activator could inhibit the stimulatory activity of a second activator in vivo (Gill and Ptashne, 1988; Triezenberg et al., 1988a; Triezenberg et al., 1988b). This notion was further corroborated by the requirement of additional activities from partially purified TFIID fractions to support activator function apart from what was then thought to be cloned TFIID, but is now known to be TATA-binding protein (TBP), the TATA binding component of TFIID (Hoffman et al., 1990; Pugh and Tjian, 1990). These observations pointed to a TBP-associated factor (TAF)-associated cofactor activity.

The existence and function of novel cofactor activities, which are distinct from TAF-associated

activity, termed "mediator" in yeast (Flanagan et al., 1991) and "upstream factor stimulatory activity" in metazoan system (Meisterernst et al., 1991) was first indicated by biochemical assays. These studies provided direct evidence that these activities are required to compensate the incapability of reconstituted transcription system with purified GTFs to achieve the optimal activity of sequence-specific activator-induced stimulation above the basal activity, which was seen in more crude system (Flanagan et al., 1991; Meisterernst et al., 1991). The attempts to further purify these activities to homogeneity were eventually succeeded in isolation of the multiprotein complexes that could indeed support activated-transcription in reconstituted transcription systems with purified GTFs (Kim et al., 1994; Malik et al., 2000).

Prior to these biochemical studies, yeast and metazoan Mediator (or Mediator-related) complexes were identified through independent studies. Early genetic analyses identified two groups of genes that are involved in transcriptional regulation (reviewed in Carlson, 1997) in vivo. The first includes suppressors of RNA polymerase B (II) (Srbs), and the other includes Gal11, Rgr1, Rox3 and Sin4 (reviewed in Carlson, 1997). The genetic screens

for suppressors of truncation mutation in the carboxyl terminal domain (CTD) of the largest subunit of pol II, *rpb1,* first identified nine genes: srb2 (Nonet and Young, 1989), srb4-6 (Thompson et al., 1993), and srb7-11 (Hengartner et al., 1995; Liao et al., 1995). As they were originally identified the proteins involved in CTD function in vivo, SRB2, SRB4, SRB5 and SRB6 proteins, together with TBP, were found in a multisubunit complex that was associated with RNA polymerase II and bound specifically to recombinant CTD protein (Thompson et al., 1993). Further characterization of this complex, termed holoenzyme, revealed that this complex could stimulate activated-transcription by GAL-VP16 (Koleske and Young, 1994) and contained all nine SRB proteins and GAL11, together with TFIIB, TFIIF and TFIIH (Barberis et al., 1995; Hengartner et al., 1995; Koleske and Young, 1994; Koleske and Young, 1995; Liao et al., 1995).

The first human Mediator complex was discovered as TRAP (thyroid receptor-associated proteins) complex (Fondell et al., 1996). This complex was isolated through its intracellular association of the ligand-bound thyroid receptor (Fondell et al., 1996). TRAP complex was later found to be equivalent, in composition, function, and action mechanism (Ito et al., 1999), to SRB- and MED-

containing Coactivator Complex (SMCC), which was isolated through independent efforts to identify a human holoenzyme complex corresponding to yeast holoenzyme (Gu et al., 1999). Other biochemical studies reported other human Mediator-related complexes including vitamin D receptor-interacting proteins (DRIP) (Rachez et al., 1998), negative regulator of activated transcription (NAT) (Sun et al., 1998), activator-recruited complex (ARC) (Naar et al., 1999) and coactivator required for Sp1 (CRSP) (Ryu and Tjian, 1999).

1.3.2. Structure of Mediator

1.3.2.1. Subunit composition

Yeast Mediator is composed of about twenty one subunits (Myers and Kornberg, 2000; Boube et al., 2002): above-mentioned nine Srbs (Srb2-11); other genetically identified subunits including Gal11, Nut1, Rgr1, Rox3, Sin4, Med3/Hrs1/Pgd1, Med9/Cse2 and Med10/Nut2 (Carlson, 1997; Myers and Kornberg, 2000); and the last seven novel proteins (Med 1,2,4,6,7,8, and 11) that were biochemically identified by their presence in the SRB/Mediator complex (Myers et al., 1998).

Metazoan Mediator complexes appeared to vary significantly in size and composition (Malik and Roeder,

2000). However, close comparison of their compositions and sizes reveals that they are essentially derived from an identical or a very similar cellular unit (Malik and Roeder, 2000). The largest human Mediator complex, TRAP/Mediator complex contains approximately twenty-five subunits (Gu et al., 1999; Malik et al., 2000; Baek et al., 2002). Among them, seventeen subunits are human orthologs of yeast Mediator components; TRAP240/SRB9, TRAP230/SRB8, TRAP220/MED1, TRAP170/RGR1, hSUR2/Gal11, TPA-inducible gene (TIG)1/PC-associated Q-rich protein(PAQ)/ARC105/MED9, TRAP95/Sin4, TRAP80/SRB4, CDK8/hSRB10, p36/MED4, hMED7, Cyclin C/hSRB11, hMED6, TBP-related-factor-proximal protein (TRFP)/SRB2, TRAP25/Med11, hSRB7, hSOH1, hNUT2/MED10 (Malik and Roeder, 2000; Boube et al., 2002) and TRAP100, p37 are considered as species specific components. Additional subunits, p97, p93, p78, p22, p12 remain to be characterized (Malik and Roeder, 2000). Any variation in composition and size of the metazoan Mediator complexes may be possibly in part due to different isolation procedures (Malik and Roeder, 2000). In addition, the intrinsic modular properties and/or different cellular states of the Mediator complexes might also lead to variable association of the components (Malik and Roeder,

2000; Myers and Kornberg, 2000), as reported in yeast Mediator (Kang et al., 2001). On the basis of compositional complexity and sizes, these various complexes can be classified into the smaller complexes including PC2, CRSP and the murine Mediator and the larger complexes including TRAP/Mediator, ARC, DRIP and NAT complexes (Malik and Roeder, 2000). In view of the notion that Mediator is a dynamic and modular organization, the smaller complexes could be considered as subcomplexes of the larger complexes as represented by PC2 and TRAP/Mediator (Malik et al., 2000) as well as CRSP and ARC-L (Naar et al., 1999; Ryu and Tjian, 1999).

1.3.2.2. Three-dimensional structure

As inferred from the comparison of their subunit composition, the recent studies by electron microscopy (EM) revealed considerable similarities between TRAP/Mediator and ARC-L as well as between CRSP and murine Mediator complex (Dotson et al., 2000; Taatjes et al., 2002). Additionally, comparison of murine Mediator and human TRAP complex hinted that structure of murine Mediator appeared to be a substructure of TRAP/Mediator (Dotson et al., 2000). Consistently, superposition of the structures of CRSP and ARC-L demonstrated that CRSP is

essentially a substructure of ARC-L (Taatjes et al., 2002). More importantly, these structural analyses revealed significant structural features of Mediators. In the holoenzyme (Mediator and RNA polymerase II) state, three domains termed head, middle and tail of Mediator were extended and wrapped around a globular polymerase, in contrast to the more compact structure of its isolated state, suggesting that Mediator undergoes conformational changes in order to complex with polymerase (Asturias et al., 1999). Subsequent EM studies of CRSP complex showed activator-specific conformations of CRSP as distinct from its structure of the isolated form. Interestingly, certain activator i.e. VP16 can induce the conformation similar to CTD bound form (Naar et al., 2002; Taatjes et al., 2002). It was suggested that activator (VP16) could substitute for, but not compete with, a potential function of the CTD in activating transcription by inducing a CTD-bound conformation in the CRSP. In this way, activator can overcome CTD-dependent regulatory mechanisms that would otherwise moderate transcription initiation. Another possibility could be that activator binding induce such structural change in the conformation of Mediator to facilitate the formation of a holoenzyme complex which help basal machinery including RNA

polymerase II to form preinitiation complex at the promoter.

1.3.3. Function of Mediator

Mediator is now considered as a sensor, integrator, and processor (Kang et al., 2001). In this view, Mediator fulfills its primary function, as originally conjectured, by interconnecting diverse gene-specific regulatory proteins to the basal transcription machinery and providing more regulatory surfaces to transcription apparatus with its modular and dynamic organization.

Three lines of evidence support this view. First of all, Mediator facilitates activated-transcription through direct interaction with a number of transcription activators including VP16 (Hengartner et al., 1995), Sp1 (Ryu and Tjian, 1999), adenovirus oncoprotein E1A (Boyer et al., 1999), and various nuclear receptors such as thyroid receptor (Fondell et al., 1996), vitamin D receptor (Rachez et al., 1998), estrogen receptor (ER) (Kang et al., 2002), peroxisome proliferator-activated receptor γ2 (PPARγ 2) (Ge et al., 2002) and hepatocyte nuclear factor 4 (HNF-4) (Malik et al., 2002). Secondly, the roles for Mediator in basal transcription have been supported by the following observations: (1)

copurification of yeast Mediator with polymerase II (Thompson et al., 1993; Kim et al., 1994; Koleske and Young, 1994), (2) association of TBP with SRB proteins (Koleseke et al., 1992; Thompson et al., 1993) (3) the requirement of Srb2 and Srb5 for stable PIC formation (Koleseke et al., 1992; Thompson et al., 1993), (4) complete shut-off of transcription from essentially the entire genome by the mutation in *srb4*, one of the core subunits of yeast Mediator (Holstege et al., 1998) and (5) TRAP/Mediator affected the basal transcription in TRAP/Mediator-depleted nuclear extract as well as activator-dependent transcription activity (Baek et al., 2002; Mittler et al., 2001). Finally, a number of genetic and biochemical analyses have suggested that yeast Mediator is constituted with three functional modules termed the Gal11, Med9/10, and Srb4 modules (Carlson, 1997; Lee and Kim, 1998; Lee et al., 1999; Han et al., 1999; Han et al., 2001; Kang et al., 2001; Boube et al., 2002). This is consistent with the three-dimensional structure reconstructed by EM study, which also suggested dynamic properties of Mediator (Asturias et al., 1999; Dotson et al., 2000). The Gal11 module, constituting the tail region, (Dotson et al., 2000), includes Gal11, Rgr1, Sin4, Med2, and Med3 (Lee and Kim, 1998; Lee et al.,

1999). As Gal11, Rgr1 and Sin4 are originally identified by genetic analyses for transcriptional regulators (reviewed in Carlson, 1997), this module is thus proposed to function mainly in receiving signals from gene-specific regulators (Lee et al., 1999; Han et al., 1999). In support of this idea, the proteins in this module were shown to be required for transcriptional activation and repression through direct interaction with activators and repressors (e.g., Gal4, Gcn4, Swi5, and Tup1) via distinct and/or common binding surfaces (Bhoite et al., 2001; Han et al., 2001; Park et al., 2000, Han et al., 1999; Lee et al., 1999; Myers et al., 1999). The middle domain of Mediator is the Med9/10 module together with the N-terminal portion of Rgr1 (Dotson et al., 2000). This module is proposed to play a role in relaying regulatory signals (Han et al., 2001; Kang et al., 2001) through physical interactions among the Mediator subunits and basal transcription machinery (Kang et al., 2001). This module is composed of two stable substructures: the Med9 submodule containing Med1, -4, and –9, as indicated in genetic analyses, is thought to be involved in repression through physical interaction with Srb10/11, whereas the Med10 submodule comprising Srb7, Med7, Med10, and possibly Nut1 may transfer activating signals to the

basal transcription machinery (Han et al., 2001; Kang et al., 2001). The head domain, Srb4 module, includes Srb2, -4, -5, -6, Rox3, Med6, -8, and -11 (Kang et al., 2001). This module is thought to function as a signal processor that directly modulates activity of polymerase II through the physical interaction with polymerase II. This idea has emerged from the following observations: (1) the reconstituted SRB4 module interacts with basal transcription machinery and enhances basal transcription in a cell-free assay without major effect on activated transcription and CTD phosphorylation (Kang et al., 2001); (2) *srb2, -4, -5, and -6* genes were originally identified as suppressors of CTD truncation (Nonet and Young, 1989; Thompson et al., 1993); and (3) the apparent physical contacts between the head domain and Pol II was shown by EM studies (Asturias et al., 1999). Mediator under certain condition contains an accessory Srb10 module composed of four Srbs, Srb8–11 (reviewed in Carlson, 1997) whose physical location within Mediator is yet unknown. This module is implicated in transcriptional repression via a modification of the polymerase II CTD prior to initiation (Hengartner et al., 1998) and through interactions with the Med9 submodule at least by a physical interaction of the Srb10 with Med1 and Med4

(Kang et al., 2001). Consistently, the depletion of the Srb10 and Srb11 pair by nutrient deprivation was associated with upregulation of a specific gene subset (Holstege et al., 1998). Moreover, the negative cofactor activity of TRAP/Mediator (Gu et al. 1999) was attributed to the enrichment of CDK8/SRB10 due to isolation procedures. This is because phosphorylation of cyclin H subunit of TFIIH by CDK8/SRB10 represses both the ability of TFIIH to activate transcription and its CTD kinase activity (Akoulitchev et al., 2000).

Regarding metazoan Mediator complex, its modular properties are not yet as clearly elucidated as yeast Mediator. However, emerging evidence indicated the modular properties of metazoan TRAP/Mediator. For example, as mentioned above, PC2 was found to be a submodule of TRAP/Mediator (Malik et al., 2000) and most subunits of CRSP are included in ARC complex (Naar et al., 1999). Moreover, the purified Mediator complex isolated from murine cells lacking Sur2 contains reduced amount of murine TRAP95 and murine TRAP100 (Stevens et al., 2002). Consistently, the TRAP100-deficient TRAP/Mediator complex derived from mouse embryonic fibroblasts of TRAP100 knockout mice also lacks TRAP95 and SUR2, with a reduced amount of CDK8/hSRB10,

suggesting that these subunits may form a submodule (Ito et al., 2002).

Another line of evidence suggesting the modular properties of metazoan Mediator is that each subunit (or group of subunits) of Mediator serves as a specific target for a distinct group of transcription factors. For example, TRAP220 is shown to be a main anchor for various nuclear receptors whereas TRAP80 serves as a specific target for p53 and VP16 (Ito et al., 1999). Human Sur2 is implicated as an integrator of the E1A and RAS signaling pathways (Boyer et al., 1999), while TRAP230 is involved in Wnt signaling (Zhang and Emmons, 2000). These studies also reinforce the idea that TRAP/Mediator, as a common and general cofactor, integrates multiple signals from diverse transcriptional activators to coordinate afferent signals from various signaling pathways.

1.3.3.1. Functional mechanism of Mediator: The relationship with chromatin remodeling factors

Transcriptional activation may be viewed as a two-step process, an antirepression process to relieve the repression imposed by high order chromatin structure followed by PIC formation at the promoter (Roeder, 1998). The observation that TRAP/Mediator does not contain

intrinsic HAT activity and functions on naked DNA template in a purified system indicates that TRAP/Mediator mainly contributes to the PIC formation step (Roeder, 1998; Malik and Roeder, 2000). Additionally, the report of the competitive interaction of TRAP220 and TIF2 with TR or PPARγ (Treuter et al., 1999) has suggested mutually exclusive recruitment of these proteins to transcription activators. Another important finding was the sequential interaction of cofactors with ligand-bound TR, TR-p160/SRC-CBP/pCAF followed by TR-TRAP (Sharma et al., 2000). These have proposed the sequential multi-step model for transcription activation (Roeder, 1998; Ito and Roeder, 2001 and references therein). In this model, transcription activation occurs in four steps: (1) activator binding to target sites within chromatin; (2) recruitment of chromatin remodeling factors including ATP-dependent nucleosome remodeling factors and HATs; (3) exchange of chromatin remodeling factors with TRAP/Mediator; (4) recruitment of RNA polymerase II and GTFs to form PIC.

A recent report provided evidence for this model showing that TR, upon ligand binding, recruited HATs such as SRC-1, GRIP-1 and p300 ahead of the TRAP220 (Sharma

and Fondell, 2002). Prior to this study, however, the contrasting observation that TRAP220/PBP and AIB1 concurrently occupied the cathepsin D promoter upon estrogen treatment had proposed the simultaneous and combinatorial recruitment model (Shang et al., 2000). The discrepancy between these studies might be attributed to the intrinsic differences between ER and TR (Sharma and Fondell, 2002). However, and more importantly, the observation of the requirement of p160/GRIP1 for TRAP220/PBP recruitment (Shang et al., 2000), actually provided evidence for the sequential action of these cofactors. Interestingly, Burakov et al suggested that TRAP220 subunit may have been recruited to the pS2 promoter earlier than the rest of TRAP/Mediator complex (Burakov et al., 2002). Taken together, these results indicated that SRC/p160 and TRAP220/PBP appeared to be recruited to the promoter simultaneously but the concrete recruitment of TRAP/Mediator complex might occur at the later step.

1.3.3.2. Physiological roles of Mediator subunit

In the multicellular organism, it is necessary to accommodate and incorporate diverse regulatory information needed to coordinate and specify the various

cell types. To fulfill this necessity, TRAP/Mediator, as a multiprotein complex, may provide a physical interface whereby subunits serve as specific targets for distinct regulatory signals. The genetic analyses in model eukaryotes have provided insights of the involvement of Mediator in the cellular mechanisms that allow the tight transcriptional regulation necessary for successful differentiation and/or development.

Three genes encoding metazoan counterpart of MED6, SRB4, and SRB7 are essential for cell viability suggesting their general function in RNA polymerase II transcription (Tudor et al., 1999; Boube et al., 2000; Gim et al., 2001). In contrast to this, some of metazoan mediator subunits play more restricted but crucial roles in transcriptional regulation. For example, disruption of the murine TRAP100 has revealed that TRAP100 is not essential for cell viability *per se*, however, null mutant mice die at an early developmental stage with severe malformations (Ito et al., 2002). Similarly, although metazoan counterparts of Gal11/Sur2, MED1/TRAP220, SRB8/TRAP230, and SRB9/TRAP240 are not required for cell viability or proliferation *per se*, these genes are necessary for the viability of the whole organism

(Stevens et al., 2002; Treisman, 2001; Boube et al., 2000; Ito et al., 2000; Zhu et al., 2000).

These subunits play their specific roles in transcriptional response to various signaling pathways, which are involved in cell differentiation through environment-directed cell fate decisions. The C. elegans Sur2/Gal11, which functions downstream of the Sur1 MAP kinase (MAPK) pathway, is involved in a Ras-mediated signal transduction pathway (*sur*, suppressor of *ras*), which plays roles in the process of vulval induction (Singh and Han, 1995). Its null mutant showed pleiotropic phenotypes including partial larval lethality and sterile adults (Singh and Han, 1995). Consistently, human Sur2 functionally interacts with MAPK-modified form of Elk1 (Boyer et al., 1999). Furthermore, disruption of murine *sur2* in embryonic stem cells leads to defective gene activation specifically by Elk1, but not by many other transcription factors (Stevens et al., 2002). These studies pointed to the conserved function of metazoan Gal11, involving transcriptional regulation, particularly, in response to MAPK-dependent cell signaling.

Srb8 and Srb9 represent another example of conservation of their specific roles in cell

differentiation in response to cell signaling pathways. In the slime mold, the protozoan counterpart of Srb9, namely AmiB, is specifically required to modify gene expression in response to nutrient depletion through its function in a cAMP-dependent signaling pathway (Kon et al., 2000). SRB8/TRAP230 and MED1/TRAP220 proteins in C. elegans were implicated to act in concert within a Mediator-related complex to block improper activity of the Wnt signaling pathway in V6 neuroblasts (Zhang and Emmons, 2001) and thus function in specification of V6 cells via the *pal-1*-dependent genetic cascade as their genes, *srb8/sop1* and *med1/sop3* were originally isolated as *sop*, suppressor of *pal-1* (Zhang and Emmons, 2000, 2001). In Drosophila, *dSrb9/TRAP240* gene was originally identified as dose-sensitive modifiers of cell identity functions of the homeotic gene *Sex combs reduced* (Boube et al., 2000). Subsequent studies suggested that dSrb9/TRAP240 and dSrb8/TRAP230 function cooperatively to mediate developmental signals in early eye differentiation (Treisman, 2001).

Another way to coordinate cellular functions is mediated via hormone signaling pathways. The TRAP220 is thought to be the pivotal subunit to mediate hormone action based on the observations of its ligand-dependent

interactions with various nuclear receptors (Yuan et al., 1998), defective TR function in TRAP220-/- mice (Ito et al., 2000) and its essential functions in PPARγ-stimulated adipogenesis but not for MyoD-stimulated myogenesis (Ge et al., 2002). These studies provide strong evidence for the roles of TRAP220 in specific cellular processes. Altogether, the above-mentioned studies strongly supported the involvement of the Mediator complex in many important decisions controlling cell differentiation and development.

1.3.4. Conservation throughout evolution

The earlier observation that metazoan activators also can function in yeast hinted a high degree of functional conservation of the transcription apparatus over eukaryotic evolution (Flanagan et al., 1991). Recent studies supported not only functional (see above) but also structural conservation of Mediator. Electron microscopy data revealed the striking similarities in the overall appearance of isolated yeast and metazoan Mediator complexes (Asturias et al., 1999; Dotson et al., 2000). In addition, all the known human Mediator subunits shared counterparts in fruit flies and worms indicating a high degree of structural conservation among metazoan

Mediator complexes (Kwon et al., 1999) (Park et al., 2001) (Boube et al., 2000). Moreover, the extensive analyses of the newly available genomic sequences from eukaryotic species led to the identification of 12 additional Mediator subunits conserved from yeast to human: Srb2/Trfp, Srb5/Med28b, Srb6/Surf5, Srb8/TRAP230, Srb9/TRAP240, Gal11/Sur2, Sin4/TRAP95, Med1/TRAP220, Med4/TRAP36, Med8/ARC32, Med9/ARC105, and Med11/TRAP25 (Boube et al., 2002). The conservation of overall subunit composition in Mediator from yeast to human suggests that the functional organization of yeast Mediator might be highly conserved in metazoan complexes (Boube et al., 2002). Interestingly, a few mediator proteins are apparently present only in yeast and/or filamentous fungi, i.e., Nut1, Rox3, Med2, and –3 (Myers and Kornberg, 2000). Conversely, some human Mediator components appear specific to metazoans and/or plants, i.e., TRAP37, TRAP100, CRSP70, ARC42, and ARC92 (Boube et al., 2002). This, together with variations in the primary sequences of individual subunits might have been diversified during evolution to accommodate the species-specific regulatory inputs emerging from novel gene-specific transcription factors in eukaryotic evolution (Boube et al., 2002).

Chapter 2

Materials and Methods

A number of experimental procedures and materials have been repeatedly used throughout this work. They are usually described just once in the chapter where they were first used without being repeated in subsequent sections, unless significant modifications occurred.

2.1. Materials and Methods for Chapter 3.

Commercial reagents.

17β-estradiol (cat# E-2257) and N-lauroyl sarkosyne (Sarkosyl,cat# L-5777) were purchased from Sigma. Fetal bovine serum (cat# 16000044)was purchased from Gibco and Calf serum (cat# 12133-500M) was from JRH.

Buffer BCn.

20 mM Tris-HCl pH7.9 at 4°C, 20% (vol/vol) glycerol, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Dithiothreitol (DTT), supplemented with n mM of KCl.

Plasmids.

Plasmids encoding glutathione S-transferase (GST)—ERβLBD (243-530) were created by inserting the corresponding PCR-generated human ERβ (hERβ) derivatives into the pGEX

vector (Pharmacia). hERβ cDNA (a generous gift by Dr. Muramatsu) was used as templates for PCR reaction. GST–ERαLBD (302-595) was generously provided by Mitsuhiro Ito.

Cell Lines.

Immortalized mouse embryo fibroblasts (MEFs) from wild type and TRAP220$^{-/-}$ mice (Ito et al., 2000) by transformation with SV40 large T antigen were provided by Chao-Xing Yuan. HeLa derived cell line that stably expresses FLAG(f)-tagged Nut2 proteins (f:Nut2) (Malik et al., 2000) was used to purify TRAP/Mediator complex.

Cell Culture.

MEFs were maintained in Dulbeco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum. HeLa cells were maintained in Joklik minimal essential medium with 10% calf serum and then transferred to DME-PO_4 medium containing 10% calf serum to achieve high density (10^6/ml).

Extract Preparation.

Nuclear extracts and S100 extracts were prepared as described previously (Dignam et al., 1983).

Immunoaffinity purification of FLAG-tagged protein-assoiciated complex.

Typically, one milliliter of nuclear extract prepared from the cell line was adjusted to 300 mM KCl with 0.05% (vol/vol) Nonidet P-40 (NP-40) and incubated with 20μl of anti FLAG M2-agarose beads (Sigma), which had been preequilibrated with BC300-0.05% (vol/vol) NP40, at 4°C for 4-6 hours with gentle rotation. After 5 times of washing with BC300-0.1% (vol/vol) NP-40, the proteins were eluted by incubation of 20μl of BC300-0.05% (vol/vol) NP-40 with 0.2 mg/ml of the FLAG peptides at 4°C for 30 minutes.

Bacterial Expression of GST fusion proteins.

GST and GST fusion proteins were expressed and purified as described previously (Burakov et al., 2000).

GST-Pull-Down Assays with Nuclear Extracts.

GST pull-down assays were performed as described previously (Rachez et al., 1998). Before incubation with HeLa nuclear extract, all glutathione-Sepharose bead-immobilized GST or GST-fusion proteins were normalized to equimolarity following quantitation by SDS-PAGE. Immobilized GST or GST-fusion proteins(20 μg) were

preincubated for 1 hour at 4°C with ligand (1 μM E_2)or carrier in binding buffer (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-KOH, pH 7.9, 20% (vol/vol) glycerol, 0.2 mM EDTA, 180 mM KCl, 1 mM DTT, 0.05% (vol/vol) NP-40, 0.5 mM PMSF) containing 1mg/ml BSA. Immobilized proteins on beads were then incubated at 4°C for 6-14 hours with 4-10 mg of HeLa nuclear extracts adjusted to 180 mM KCl, plus 1 μM E_2 or carrier (ethanol). After 5 times of washing with 1 ml of washing buffer (20 mM HEPES-KOH, pH 7.9, 20% (vol/vol) glycerol, 0.2 mM EDTA, 180 mM KCl, 1 mM DTT, 0.1% (vol/vol) NP-40, 0.5 mM PMSF), bound proteins were eluted by incubating the beads with washing buffer containing 0.2% (vol/vol) sarkosyl. The eluates were subjected to 4-20% gradient SDS-PAGE, and then analyzed either by silver staining with Rapid Ag Stain Kit (ICN) or by western blot with ECL (Amersham) according to manufacturer's instructions.

Preparation of proteins for Mass Spectral Analysis.

For mass spectral analysis, eluates from GST-pull down with were subject to SDS-PAGE followed by Zinc staining (BioRad) according to manufacturer's instruction. The polypeptides were extracted from the gel and analyzed by

MS/MS by Dr. Wenzhu Zhang at Brian Chait's Lab. The MS/MS data were analyzed by Masscot.

2.2. Materials and Methods for Chapter 4.

Plasmids.

GST–ERαAB (1-180) and GST–ERβAB (1-153) were created by inserting the corresponding PCR-generated human ER (hER) derivatives into the pGEX vector (Pharmacia). hERα cDNA (purchased from ATCC) was used as template for PCR reaction. The plasmids for ER baculoviral expression were created by subcloning FLAG-tagged hERα(full-length cDNA) and hERβ(full-length cDNA) into pVL1392 vector (Invitrogen).

4EREΔ53 was generously provided by Dr. Chengming Chiang (Wu et al., 1999).

Baculoviral Expression of Recombinant ER proteins.

Recombinant FLAG-tagged ERs were expressed as described previously (Wu et al., 1999) with slight modifications. Briefly, 1 μg of each plasmid was incubated with 0.25 μg of Bac3000 linear DNA (Novagen), 1 μl of cationic liposome solution (Novagen), and 0.5 ml of Grace's medium. The mixture was vortexed vigorously, left at room

temperature for 15 min, and then added to a 60-mm plate containing ~2 x 10^6 Sf9 cells. After incubation for 4 hours, 1.5 ml of TNM-FH was added to the plate. Incubation was continued in a 27 °C humidified chamber for 5 days. The supernatant, collected after pelleting cells at 3,000 rpm for 5 min, was designated as the P0 virus stock. 0.5 ml of P0 was incubated with ~4x10^5 Sf9 cells in a 60-mm plate containing 3 ml of TNM-FH. After 5 days, the supernatant (P1 virus, 0.5 ml) was used to infect 6x10^6 Sf9 cells in a 150-mm plate containing 25 ml of TNM-FH. The supernatant (P2 virus, 5 ml), collected after 5 days of incubation, was used to infect 250 ml (~0.6x10^6 cells/ml) of Sf9 cells in suspension. Fifty ml of the final P3 virus stock, collected after a 5-day incubation, was then used to infect 500 ml (1 x10^6 cells/ml) of Sf9 cells for protein production, which was conducted for 48 to 60 hours. In the interim, estradiol, if included, was added to the medium at a final concentration of 10 nM, 16 h before harvest.

Purification of Recombinant ER proteins.

To purify FLAG-tagged ER, cells were collected by centrifugation at 3000 rpm for 10 minutes in a JS 4.2 rotor at 4 °C. After washing with cold phosphate-buffered

saline (PBS) nuclear extract was prepared as described previously (Dignam et al., 1983) with modifications. Instead of adding low salt buffer and high salt buffer separately, nuclear extract buffer (20 mM Hepes, pH 7.9, 25% (vol/vol) glycerol, 0.2 mM EDTA, 420 mM NaCl, 1 mM DTT, 0.05% (vol/vol) NP-40, 0.5 mM PMSF, 1mM Benzamide, 1mM Na_3VO_4, 1μM E_2) was used to extract nuclear proteins. 14 ml of the nuclear extract was incubated with 0.1 ml of anti-FLAG M2-agarose (Sigma) at 4 °C for 4 to 6 hours. The immobilized proteins were then washed 5 times with 10 ml of BC600-0.1% (vol/vol) NP-40 followed by washing with BC100-0.05% (vol/vol) NP-40 and finally eluted with elution buffer (BC100 for the first elution, and BC300 for the next elution) containing 0.2 mg/ml FLAG peptide and 0.05% (vol/vol) NP-40.

Cell lines.

HeLa-derived cell lines expressing FLAG(f)-tagged Thyroid hormone receptor (f:TR) (Fondell et al., 1996), f:CDK8/SRB10 (Gu et al., 1999), f:TRAP220AB(1-670) (C.X.Y. and R.G.R, unpublished) were used to purify the corresponding TRAP/Mediator complexes containing these FLAG-tagged proteins. Chao-Xing Yuan established a HeLa-derived cell line (C8) that stably expresses f:hERαΔAB

(160-595), which was used to purify the hERαΔAB-associated proteins.

Antibodies and Western Blot Analysis.

Antibodies against TRAP/Mediator subunits were described previously (Yuan et al., 1998) (Ito et al., 1999) (Malik et al., 2000). Western blot analyses involved standard procedures with an enhanced chemiluminescence detection kit (Amersham) according to manufacturer's instruction.

GST-pull down assays with purified proteins.

GST pull-down assays were performed as described in the previous section. Instead of nuclear extracts the purified complex supplemented with 2 mg/ml of BSA was incubated with immobilized proteins on beads at 4°C for 6-14 hours under the same binding condition then processed and analyzed as described previously.

Immunoaffinity pull down assays.

All anti FLAG M2-agarose bead-immobilized f:ER proteins were normalized to equimolarity following quantitation by SDS-PAGE. Immobilized f:ER proteins (10 μg) on beads as well as the same volume of empty M2-agarose beads were preincubated for 1 hour at 4°C with ligand (1 μM E_2)or

carrier in binding buffer (20 mM HEPES-KOH, pH 7.9, 20% (vol/vol) glycerol, 0.2 mM EDTA, 180 mM KCl, 1 mM DTT, 0.05% (vol/vol) NP-40, 0.5 mM PMSF) containing 1mg/ml BSA. The beads were then incubated at 4°C for 6-14 hours with 8-10 mg of HeLa nuclear extracts adjusted to 180 mM KCl, plus 1 μM E_2 or carrier. After 5 washes with 1 ml of washing buffer (20 mM HEPES-KOH, pH 7.9, 20% (vol/vol) glycerol, 0.2 mM EDTA, 180 mM KCl, 1 mM DTT, 0.1% (vol/vol) NP-40, 0.5 mM PMSF) bound proteins were eluted by incubating the beads with washing buffer containing 0.2 mg/ml of FLAG peptides. The eluates were subjected to 4-20% gradient SDS-PAGE, and then analyzed by western blot with ECL (Amersham) according to manufacturer's instructions.

In vitro translation of ER.

^{35}S-labelled ERβ was expressed by TNT T7 coupled reticulocyte lysate systems (Promega cat#L4610) according to manufacturer's instructions.

Interactions of purified TRAP/Mediator and full-length ER.

TRAP/Mediator complex purified from the cell line expressing f:TRAP220AB was immobilized on anti FLAG M2-

agarose beads. Translation mixture containing the expressed full-length ER and mock translation mixture were preincubated for 1 hour at 4°C with ligand (1 μM E_2)or carrier in binding buffer (20 mM HEPES-KOH, pH 7.9, 20% (vol/vol) glycerol, 0.2 mM EDTA, 180 mM KCl, 1 mM DTT, 0.05% (vol/vol) NP-40, 0.5 mM PMSF). Immobilized TRAP/Mediator on beads as well as the same volume of control empty M2-agarose beads were then incubated with each translation mixture at 4°C for 6-14 hours in the binding buffer with 1 mg/ml of BSA plus 1 μM E_2 or carrier. After washing 5 times with 1 ml of washing buffer (20 mM HEPES-KOH, pH 7.9, 20% (vol/vol) glycerol, 0.2 mM EDTA, 180 mM KCl, 1 mM DTT, 0.1% (vol/vol) NP-40, 0.5 mM PMSF) bound proteins were eluted by incubating the beads with washing buffer containing 0.2 mg/ml of FLAG peptides. The eluates were subjected to 4-20% gradient SDS-PAGE, and then analyzed by western blot with ECL (Amersham) according to manufacturer's instructions.

In Vitro Transcription Assays.

In vitro transcription assays were done by Mohamed Guermah. Reactions contained TFIID, TFIIB, TFIIE, TFIIF, TFIIH, RNA polymerase II, PC4 and other components (TRAP/Mediator and ERs) as indicated. All factors were

either recombinant or natural affinity-purified components that were isolated and utilized under previously described conditions (Guermah et al., 2001).

2.3. Materials and Methods for Chapter 5.

Plasmids

F:GAL4-ER LBDs were created by exchanging p53 of f:GAL4-p53 (Thut et al., 1995) with ER LBDs of GST-ER LBDs followed by subcloning into FASTBAC vector (Gibco). G5Δ53 described previously (Kundu et al., 2000) was kindly provided by Woojin An. In transient transfection assays the reporter construct was 3ERE-Xp-Luciferase construct provide by Chao-Xing Yuan contained 3 ERE sites and adenovirus major late core promoter in pGL2 vector (Promega). pHEG0-Hyg(ATCC) and pCXN2-ERβ from Dr. Muramatsu were used for mammalian expression of ERα and ERβ respectively. All TRAP220 constructs were generously provided by Chao-Xing Yuan.

Cell lines.

HeLa-derived cell lines expressing FLAG(f)-tagged TRAP220AB (1-670),f:TRAP220a,f:TRAP220b.f:TRAP220ab (C.X.Y. and R.G.R, unpublished) were used to purify the

corresponding TRAP/Mediator complexes containing these FLAG-tagged proteins. TRAP220AB contains N-terminal part of wild type TRAP220 (1-670). TRAP220a is the same N-terminal fragment with point mutation (LXXLL-LXXAA) in the first LXXLL motif, TRAP220b is the one with point mutation (LXXLL-LXXAA) in the second LXXLL motif and TRAP220ab is the one with double mutation in both LXXLL motives.

TRAP/Mediator-depleted Nuclear Extracts.

TRAP/Mediator-depleted nuclear extracts were provided by Hwa Jin Baek and Xiaoting Zhang. Hwa Jin Baek used antibody against 25-1 for immunodepletion of TRAP/Mediator from HeLa nuclear extract as described previously (Baek et al., 2002) and Xiaoting Zhang used antibody against TRAP220 (Xiaoting Zhang and RGR, unpublished results) for depletion of TRAP/Mediator from Namalwa nuclear extract.

Transient transfection assays.

Transfection was carried out using FuGENE 6 reagent (Roche) according to manufacturer's instructions with slight modification. MEF cells were plated in 24-well plates ($5x10^4$ cells/well) in phenol red-free DMEM with

10% charcoal-stripped fetal calf serum. The following day transfection was carried out using FuGENE 6 reagent (Roche) with 0.35 μg of DNA comprising 1-5 ng of estrogen receptor expression vector, 250 ng of luciferase reporter vector, and 100 ng of TRAP220 or empty vector and pRL-CMV for internal control. After incubation at 37 °C for 5 hours cells were treated with either E_2 or carrier. After another 36 hours cells were harvested and assayed for luciferase activity by Dual Luciferase Assay system (Promega) according to manufacturer's instruction.

Baculoviral Expression of GAL4-ER LBDs.

GAL4-ER LBDs were expressed and purified as described in the materials and methods for chapter 4.

Purification of General Transcription Factors.

TFIIB (Ge et al., 1996), TFIIE (Ohkuma et al., 1995), TFIIF (Wang et al., 1994) and PC4 (Ge et al., 1996) were expressed and purified as described previously . TFIID was purified from the cell line expressing f:TBP (3-10) (Chiang et al., 1993) as described previously (Ge et al., 1996). TFIIH and RNA polymerase II were immunoaffinity purified from the cell lines expressing f:ERCC3 (C3a) and

f:RPB9(B9b) respectively (unpublished materials by Chao-Xing Yuan).

In Vitro Transcription Assays with nuclear extract.

In vitro transcription assays with nuclear extracts were carried out essentially as described previously (Dignam et al., 1983). Transcription reactions were performed in a final volume of 25 μl. Transcription reaction contained the indicated factors (such as GAL4 fusion proteins and TRAP/Mediator complex), 5 μl (~50 μg of total proteins) of HeLa nuclear extract, 35 ng of each supercoiled plasmid DNA template, 20 mM HEPES-KOH, pH7.9, 12% (vol/vol) glycerol, 60 mM KCl, 4 mM $MgCl_2$, 0.5 mg/ml BSA, 8 mM DTT, 0.5 mM ATP and CTP, 0.125 mM UTP, 0.1 mM 3'-O-Met-GTP, 10 μCi ^{32}P UTP, 40 U of recombinant RNasin (Promega), Activator and DNA templates were preincubated at room temperature for 10 to 15 minutes followed by addition of nuclear extracts and another 20 minute-incubation on ice. Transcription was initiated by addition of NTPs and processed and analyzed as described.

In Vitro Transcription Assays reconstituted system with purified factors.

In vitro transcription assays with reconstituted system with purified factors were carried out essentially as described previously (Malik et al., 2000). Transcription reaction included the same components as described above except 5 ng of TFIIB, 7.5 ng of TFIIE, 10 ng of TFIIF, 75 ng of PC4, TFIID equivalent to 2 ng of TBP, 20 ng of TFIIH, 50 ng of RNA polymerase II instead of nuclear extracts and other components (TRAP/Mediator and ERs) as indicated. Activator and DNA templates were preincubated at room temperature for 10 to 15 minutes followed by addition of general transcription factors and another 20 minute-incubation on ice. Transcription was initiated by addition of NTPs and processed and analyzed as described.

2.4. Materials and Methods for Chapter 6.

Antibodies and Western Blot Analysis.

Antibody against EDD/hHYD was kindly provided Dr. Watts (Callaghan et al., 1998). Western blot analyses involved standard procedures with an enhanced chemiluminescence detection kit (Amersham) according to manufacturer's instruction.

In vitro translation of EDD/hHYD derivatives

^{35}S-labelled various EDD/hHYD derivatives from the constructs provided by Dr.Watts (Callaghan et al., 1998) were expressed by TNT T7 coupled reticulocyte lysate systems (Promega cat#L4610) according to manufacturer's instructions.

Transient transfection assays.

Transfection was carried out using FuGENE 6 reagent (Roche) according to manufacturer's instructions with slight modification. CV1 and Ishikawa cells were plated in 24-well plates ($5x10^4$ cells/well) in phenol red-free DMEM with 10% charcoal-stripped fetal calf serum. At the following day, transfection was carried out using FuGENE 6 reagent (Roche) with 0.75 μg of DNA comprising 1-5 ng of estrogen receptor expression vector, 250 ng of luciferase reporter vector, and indicated amount of the plasmids containing EDD/hHYD full-lenth or empty vector and pRL-CMV for internal control. After incubation at 37 °C for 5 hours cells were treated with either E_2 or carrier. After another 36 hours cells were harvested and assayed for luciferase activity by Dual Luciferase Assay system (Promega) according to manufacturer's instruction.

Chapter 3

Identification of ER-interacting Proteins

3.1. Introduction

Due to the clinical and physiological importance of estrogen and SERMs, the molecular mechanism of ER-mediated gene expression has received increased attention and becomes one of the most studied subjects. As a result, many diverse molecular mechanisms of ER-mediated gene expression have been suggested and numerous factors have been shown to be involved in those processes (see above). In 1994, an apparent involvement of cofactors in ER function emerged from the discovery of ERAPs (Halachmi et al., 1994). In 1996, the striking discovery of the second ER, which is encoded by a different gene and exhibits dissimilarity with ERα in many aspects including its primary structure (Kuiper et al., 1996), revealed another layer of complexity of ER action and led to a reevaluation of ER action in estrogen signaling.

Although the mechanisms that regulate ER functions appear to be complex and diverse, they eventually could be integrated into one that modulates the communication properties of ERs. Given that ERs, as components of huge multiprotein transcription machinery, communicate with other factors via direct and/or indirect interactions, the actions of ER is the result of the interplay by all

the factors that are involved. The primary factors that mediate and modulate this communication process, by integrating the different signals and diversifying the regulatory controls, are thought to be cofactors (see above). In support of this notion, the first crystal structures of ERα LBD complexed with E_2 and RAL demonstrated that a discrete class of ligands induced distinct conformations in the AF-2 domain that is responsible for interaction with cofactors (Brzozowski et al., 1997). This suggested that the discrete properties of ligands, through characteristic structures of ERs, eventually led to the modulation of interactions with cofactors.

Taken together, I set up the hypothesis that ERs might recruit different repertoires of ER-interacting proteins (presumptive cofactors) depending on the ligands, the subtypes of ERs, and the cell/tissue types. Furthermore, this different repertoire of ER-interacting proteins could be one of the primary mechanisms to impose specificity of ER-mediated gene expression and eventually to explain the actions of SERMs.

Here I describe my attempt to identify and compare the repertoire of nuclear proteins that interact with ERs depending on ligand, subtypes of ER and cell-types. I

employed GST pull down assays based on two earlier successful studies with nuclear receptors that: 1) identified ER interacting proteins such as ERAP 160 and ERAP 140 depending on the ligand (Halachmi et al., 1994) and 2) identified large groups of proteins such as DRIP complex (Rachez et al., 1998). To examine the subtype specificity, I decided to use GST-fused ERα full-length and GST-ERβ full-length. The rationale for employing the full-length proteins was not only to detect both AF-1- and AF-2- interacting proteins but also to avoid a simple reproduction of the previous report (Halachmi et al., 1994). In an effort to examine cell-type specificity, I chose three representative cell lines: an MCF-7 breast cancer cell line in which TAM works as an antagonist, Ishikawa endometrial cancer cells in which TAM works as an agonist, and HeLa uterine cervical cell line as an ER negative cell.

In this study, there seemed to be no major significant differences among three cell-types in the overall electrophoresis migration patterns of polypeptides interacting with GST-ERα LBD and GST-ERβ full-length in the presence of E_2. A number of HeLa nuclear proteins interacting with GST-ER LBDs in the presence of E_2 were isolated and identified. These

included TRAP/Mediator complex, which bound to both GST-ERα LBD and GST-ERβ LBD and several other proteins, which bound exclusively to GST-ERα LBD independently of TRAP/Mediator.

3.2. Results

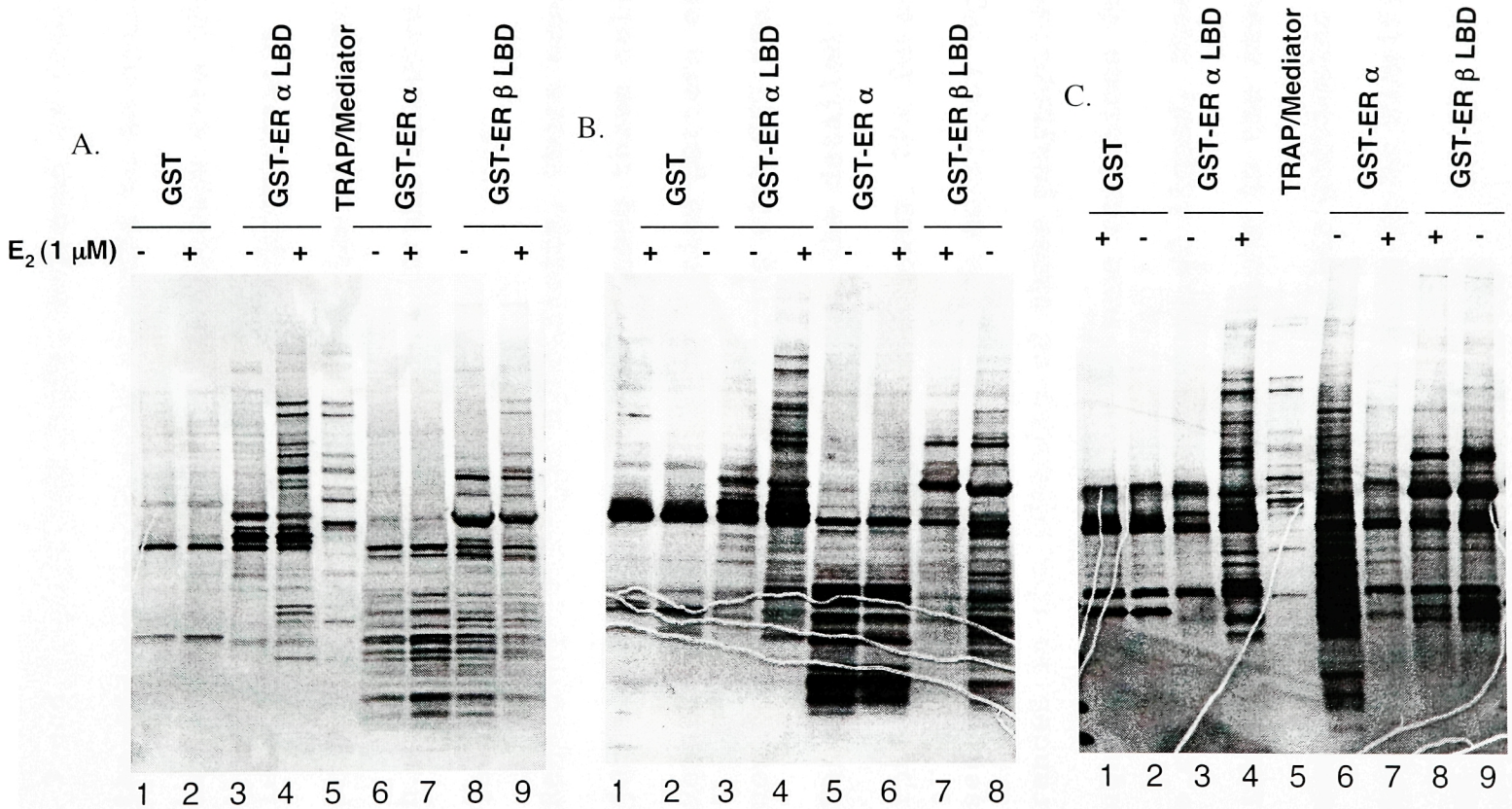
3.2.1. Purification of ER interacting proteins

The GST and GST-fused ERα full-length, ERα LBD (residues 302-595) and GST-ERβ full-length were expressed, purified and immobilized on glutathione-Sepharose beads as described in the Materials and Methods. After incubation with nuclear extracts derived from MCF-7 (Fig. 3-1A) or Ishikawa (Fig. 3-1B) or HeLa (Fig. 3-1C) cells in the presence or absence of 1 μM E_2, beads were washed extensively and bound proteins eluted and analyzed by SDS-PAGE followed by silver staining.

As shown in Fig 3-1, a large number of polypeptides (circa 30 to 40 in each case) were found to interact with GST-ERα LBD and GST-ERβ full-length in the presence of 1 μM E_2 (lanes 4 and 9 in Fig. 3-1A and 3-1C, lanes 4 and 8

Fig. 3-1. E_2-dependent interactions of nuclear extract proteins with ERα full-length, ERα LBD and ERβ full-length. Nuclear extracts were derived from MCF-7 (A), Ishikawa (B) and HeLa (C) cell lines. Immobilized GST (lanes 1 and 2), GST-ERαLBD (lanes 3 and 4) and GST-ERβLBD (lanes 8 and 9 in A and C, lanes 7 and 8 in B) proteins were incubated with nuclear extract in the absence (-) or presence (+) of 1 μM E_2 and bound proteins were eluted and analyzed by SDS-PAGE and silver staining as described in Materials and Methods. Purified TRAP/Mediator complex from f:Nut2 expressing cells was analyzed in lane 5 in A and C.

E_2-dependent interactions of nuclear extract proteins with ER LBDs



in figure 3-1B). There were a number of polypeptides interacting with GST-ERα LBD and GST-ERβ full-length in the absence of E_2 (lanes 3 and 8 in Fig. 3-1A and 3-1C, lanes 3 and 7 in figure 3-1B), but most of them appeared with the GST alone and thus appeared to be nonspecific. The results of the GST-pull down assays with GST-ERα full-length were not easy to interpret clearly. This was most probably due to the degradation of GST-ERα full-length proteins, which was seen in the isolated GST-ERα full-length proteins alone.

Regarding cell-type specificity, there seemed to be no major significant difference among three cell-types in the overall electrophoretic migration pattern of the prominent polypeptides interacting with GST-ERα LBD in the presence of E_2 (see below for the detailed description in case of HeLa). However, the further analyses are required to establish cell-type dependent differences in the identity of these polypeptides. Although there appeared to be some variations depending on the cell types in the absence of ligand, these were most likely due to slight variations in the amounts of GST fused proteins used in each this particular experiment. Again, this also needs to be clarified by further studies.

Concerning subtype specificity, direct comparisons were not possible due to difficulty in interpreting the results with the GST-ERα full-length. However, comparison of the results with GST-ERα LBD and GST-ERβ full-length revealed that circa twenty polypeptides were found to interact with both GST-fused proteins in the presence of E_2 although they showed stronger interactons with GST-ERα LBD than with GST-ERβ full-length. Interestingly, there were circa 15 polypeptides bound, seemingly specifically, to GST-ERα LBD in the presence of E_2. The results with GST-ERα LBD were different from the previous reports using a similar assay identified either only two proteins or almost nothing (Halachmi et al., 1994; Rachez et al., 1998). This difference might be due to different constructs of GST-ERα LBD [302-595 in this study v.s. 312-595 in the previous study (Rachez et al., 1998)] and probable variation of assay condition [although I followed the procedure as described previously (Rachez et al., 1998)]. For further characterization of this preliminary result, I decided to use HeLa nuclear extract because of practical reasons and GST-ER LBDs because of the failure to get clear results with the GST-ERα full-length. Thus, I generated a corresponding ERβ LBD

(residues 243-530) fused to GST to compare with the GST-ERα LBD for receptor subtype specificity.

3.2.2. A number of nuclear proteins interact with ER

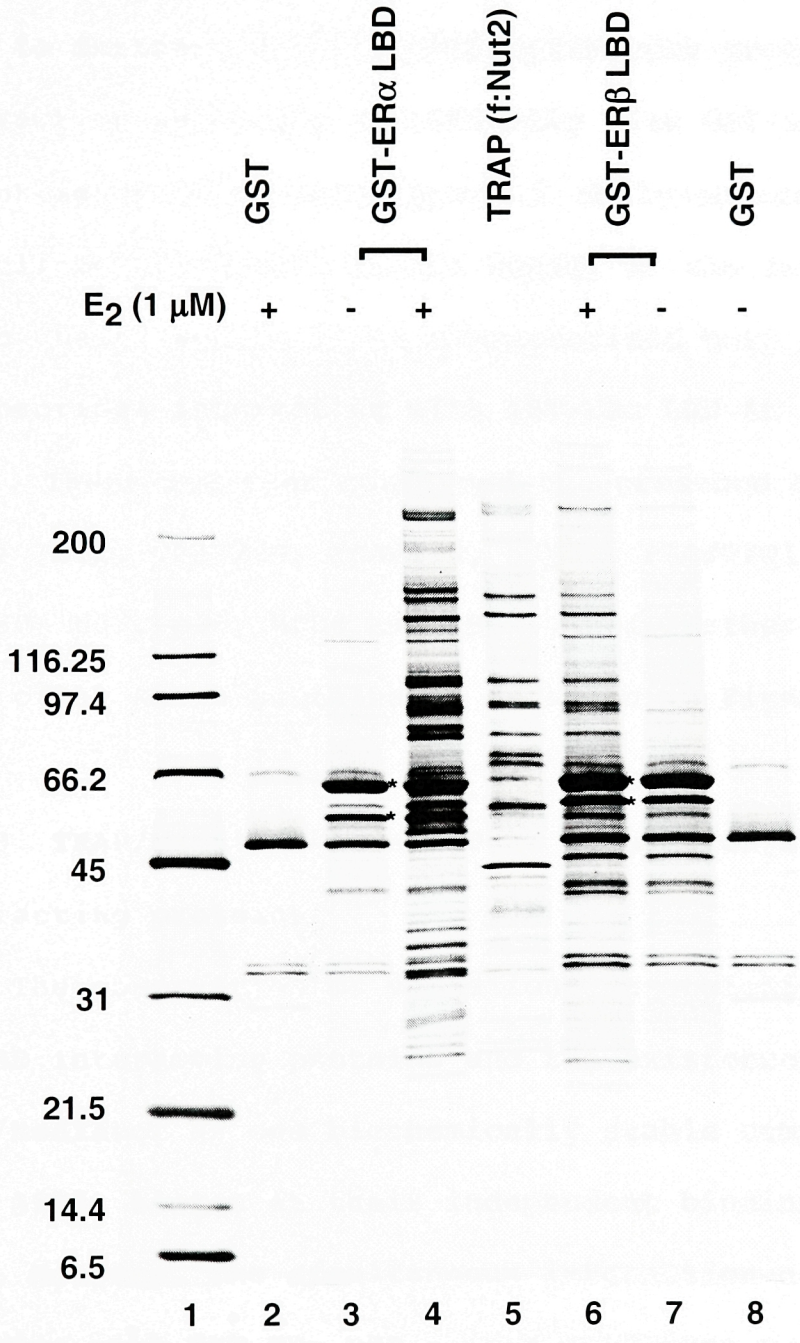
To characterize the proteins bound to GST-ER LBDs, GST-pull down assays were carried out essentially as described above except GST-fused ERα (residues 302-595) and ERβ (residues 243-530) LBDs were used with HeLa nuclear extract in the presence and absence of E_2.

As shown in Fig. 3-2, circa 5 proteins bound specifically but quite weakly to GST-ERα LBD (lane 3) and circa 10 proteins bound specifically but weakly to GST-ERβ LBD (lane 7), relative to GST alone, in an E_2-independent manner (lanes 3 and 7); and these proteins largely (but not completely) overlapped for ERα and ERβ.

Much larger groups of proteins (circa 30-40 in each case) showed E_2-dependent interactions with the GST-LBDs (lanes 4 and 6) and these also largely (but not completely) overlapped for ERα and ERβ. Many of the polypeptides commonly associated with both GST-ER LBDs in the presence of E_2 appeared similar in size to components of the circa 25-subunit TRAP/Mediator complex. This was further indicated by a direct comparison of the independently purified TRAP/Mediator complex (which

Fig. 3-2. E_2-dependent interactions of HeLa nuclear extract proteins with ERα and ERβ LBDs. Immobilized GST (lanes 2 and 8), GST-ERαLBD (lanes 3 and 4) and GST-ERβLBD (lanes 6 and 7) proteins were incubated with HeLa nuclear extract in the absence (-) or presence (+) of 1 μM E_2 and bound proteins were eluted and analyzed by SDS-PAGE and silver staining as described in Materials and Methods. Purified TRAP/Mediator complex from f:Nut2 expressing cells was analyzed in lane 5. Standard molecular weight markers with sizes in kDa indicated on the left were present in lane 1. Bands marked with an asterisk represent degradation products of GST-ER fusion proteins.

E_2-dependent interactions of HeLa nuclear extract proteins with ERα and ERβ LBDs



contains some polypeptides that non-specifically bind to M2 agarose; and lane 2 in Fig. 4-8A) and subsequent analysis of TRAP/Mediator subunit interactions with the LBDs by western blot (see chapter 4).

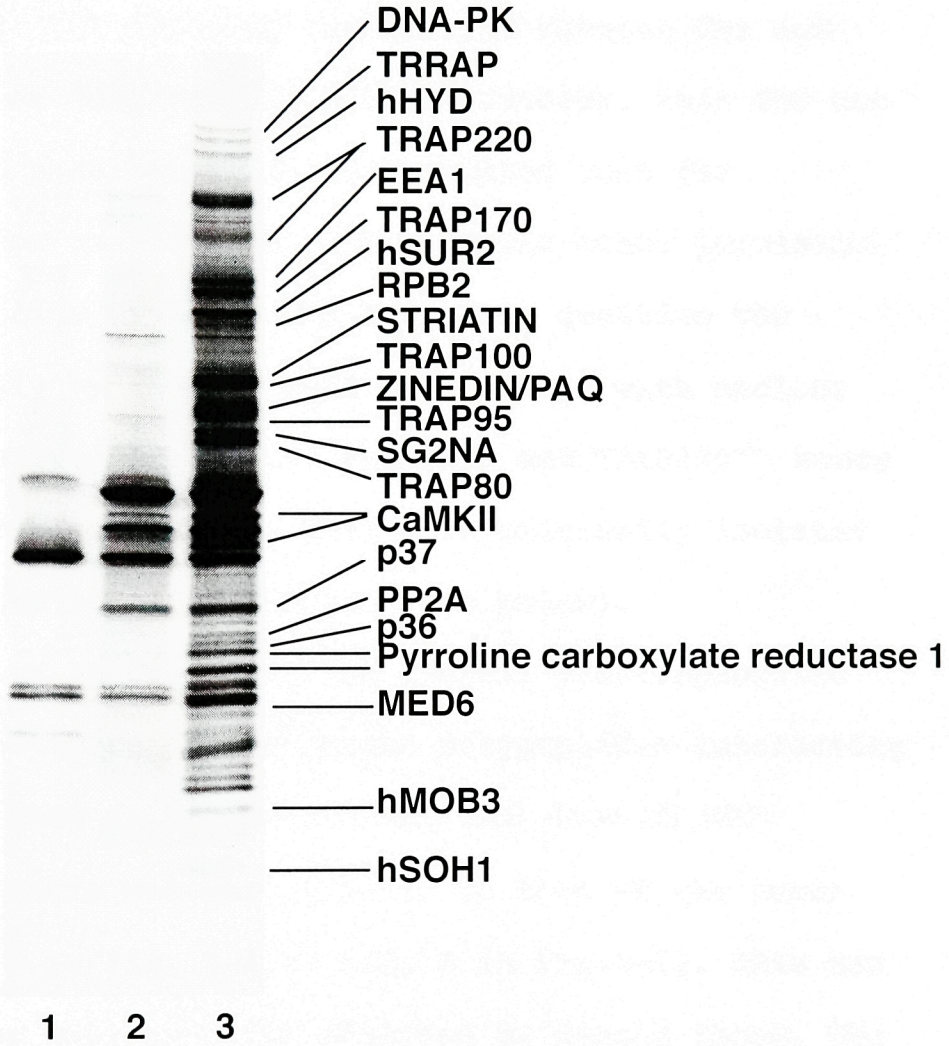
To further identify other prominent proteins interacting seemingly specifically with GST-ERα LBD in the presence of E_2, mass spectral analyses was employed in collaboration with Wenzhu Zhang, at the laboratory of Brian Chait. Wenzhu Zhang characterized most of the polypeptides interacting with GST-ERα LBD in the presence of E_2. These analyses confirmed the presence of specific TRAPs (e.g. TRAP220, TRAP170, hSUR2, PAQ/TIG1, TRAP95, TRAP80, p37, p36, MED6 and hSOH1) and further revealed many other known proteins as detailed in Figure 3-3.

3.2.3. TRAP/Mediator-independent population(s) in ER LBD-interacting proteins

The observation of variations in stoichiometry of ER-LBD interacting proteins and the existence of TRAP/Mediator as one biochemically stable complex (Gu et al., 1999) hinted at their independent binding to ER LBDs. However, the simultaneous interaction of these proteins with GST-ERα LBD raised questions regarding the possible cooperativity in their interactions and

Fig. 3-3. Composition of E_2-dependent ERα LBD-interacting proteins. Identity of proteins is determined by mass spectral analysis.

E2-dependent ERα LBD Interacting Proteins

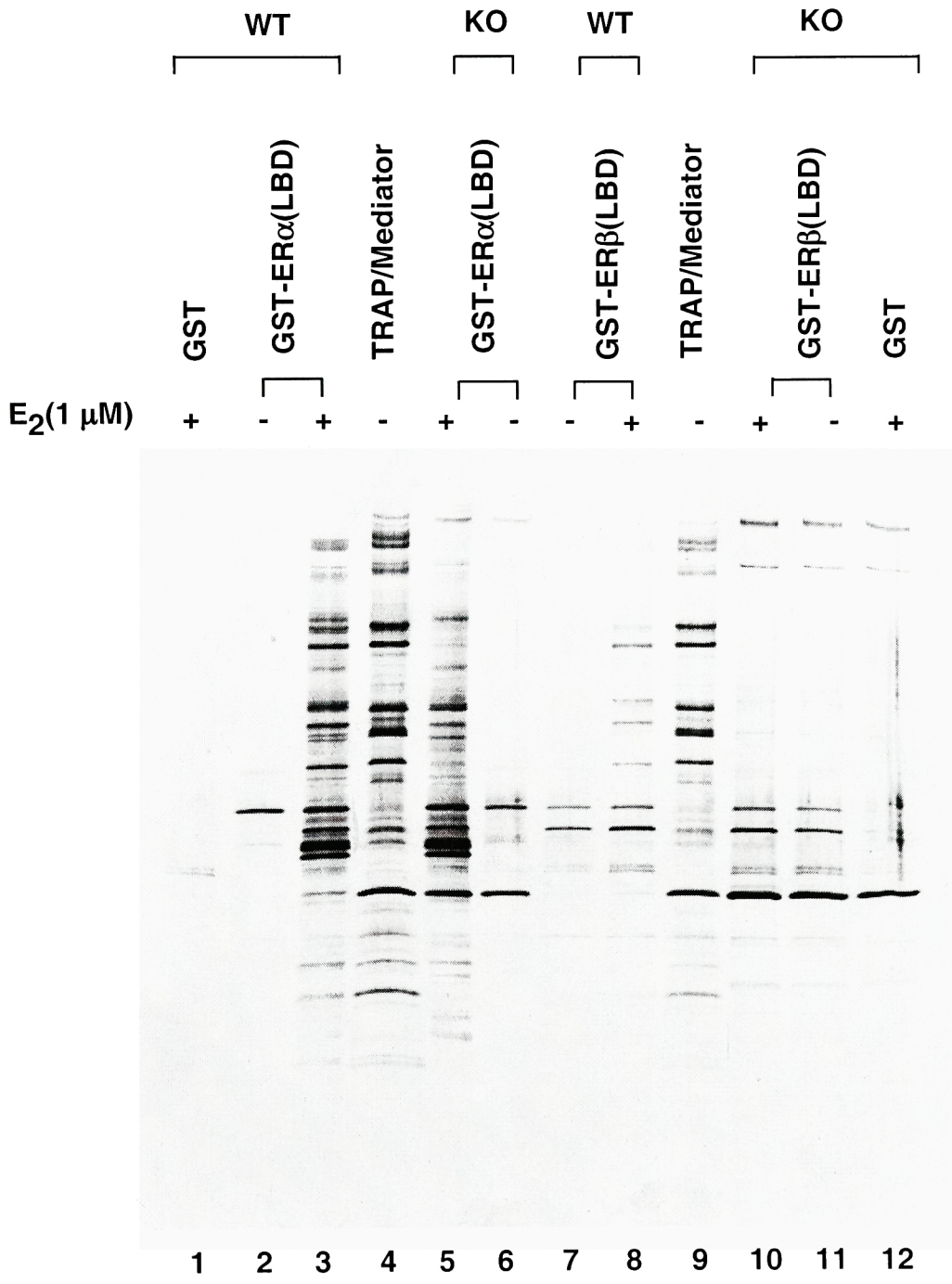


functions: 1) Does TRAP/Mediator interact with GST-ERα LBD directly or through other proteins in the total population or vice versa? ; 2) Is the interaction of TRAP/Mediator enhanced by other proteins in the population or vice versa? Independent binding rather than interdependent binding was suggested by further analyses showing a direct physical interaction between ERs and TRAP/Mediator complex (see below). However, this did not exclude the possibility of an essential role for TRAP/Mediator in the interaction of the other population of the proteins with GST-ERα LBD. This question was solved partly by the GST-pull down assays with nuclear extracts derived from wild type (WT) and TRAP220$^{-/-}$ mouse embryo fibroblasts (MEFs) that were originally isolated to assess the role for TRAP220 (see below).

As shown in Fig. 3-4, the overall electrophoretic migration patterns of the murine polypeptides interacting in the presence of E_2 with GST-ERα LBD from WT MEF nuclear extracts was very similar to that of the human ones (lane 3 in Fig. 3-4 vs lane 4 in Fig.3-2). This was confirmed by mass-spectral analyses by Wenzhu Zhang. The analyses with TRAP220-/- MEF-derived nuclear extracts revealed that most of the polypeptides other than TRAP/Mediator retained on GST-fused ER LBDs in an E_2-

Fig. 3-4. TRAP/Mediator-independent interactions of proteins with ER LBDs in nuclear extract. Immobilized GST (lanes 1 and 12), GST-ERα LBD (lanes 2, 3, 5, and 6) and GST-ERβ LBD (lanes 7, 8, 10,11) were incubated with the nuclear extracts from wild type (WT) (lanes 1, 2,3,7 and 8) and TRAP220$^{-/-}$(KO) MEFs (lanes 5,6,10,11 and 12) in the absence (-) or presence (+) of 1 μM E_2 and bound proteins were eluted and analyzed by SDS-PAGE and silver staining as detailed in Materials and Methods. TRAP/Mediator complexes immunopurified from cells expressing f:Nut2 are shown lanes 4 and 9.

TRAP/Mediator-Independent ER-interacting proteins



dependent manner, thus indicating that these polypeptides bound to GST-ER LBDs independently of TRAP/Mediator complex. Moreover, the observation that the intensity of the interaction was not affected by the absence of TRAP/Mediator complex also suggested independent binding rather than cooperativity.

Chapter 4

The TRAP/Mediator Coactivator Complex Interacts Directly with ER α and ER β and Directly Enhances ER Function In Vitro

4.1. Introduction

As mentioned in chapter 3, as a part of a broader effort to identify novel ER-interacting factors (presumptive cofactors) that might show specificity for ERα and β subtypes and/or mediate tissue selective functions of SERMs (reviewed in McDonnell, 1999), I found E_2-dependent interactions of the complete TRAP/Mediator complex with both ERα and ERβ.

TRAP/Mediator complex was first identified through a ligand-dependent intracellular association with thyroid hormone receptor (TR) and shown to be essential for TR function on DNA templates in a reconstituted cell free system (Fondell et al., 1996). Subsequent isolation of the DRIP complex, apparently identical to TRAP/Mediator complex, extended its specificity for VDR through a ligand-dependent interaction with GST-VDR LBD (Rachez et al., 1998). However, the functional involvement of TRAP/Mediator complex in the action of class I (steroid hormone) nuclear receptors was not as well established as class II nuclear receptors such as TR and VDR. Despite the strong body of evidence indicating the significance of TRAP220 subunit in ER function (see below), it was suspected that ER might not interact with or coactivate

through intact TRAP/Mediator complex because of the following; (1) a failure to detect interaction of DRIP complex with ERαLBD in their GST-pull down assays (Rachez et al., 1998), (2) subsequent failures to demonstrate the interaction between ERs and the intact TRAP/Mediator complex by Freedman and his colleagues (Burakov et al., 2000) and by Chao-Xing Yuan (unpublished observation) using immunoaffinity purification assays from the cell lines expressing epitope tagged ER alpha. In fact, one of the latter studies suggested that ER might function through TRAP220 alone or through a different TRAP220 (sub)complex (Burakov et al., 2000).

The study described here firmly established the role for the intact TRAP/Mediator complex in ER dependent transcription by showing that the interactions between TRAP/Mediator and ERs are direct and dependent on the ER LBD (but apparently modulated by the AF-1 domain) and that TRAP/Mediator can directly facilitates ER function in a cell-free system. This was the first report on the functional involvement of the intact TRAP/Mediator complex in the actions of class I (steroid hormone) nuclear receptors and further supported the notion that TRAP/Mediator is the general cofactor.

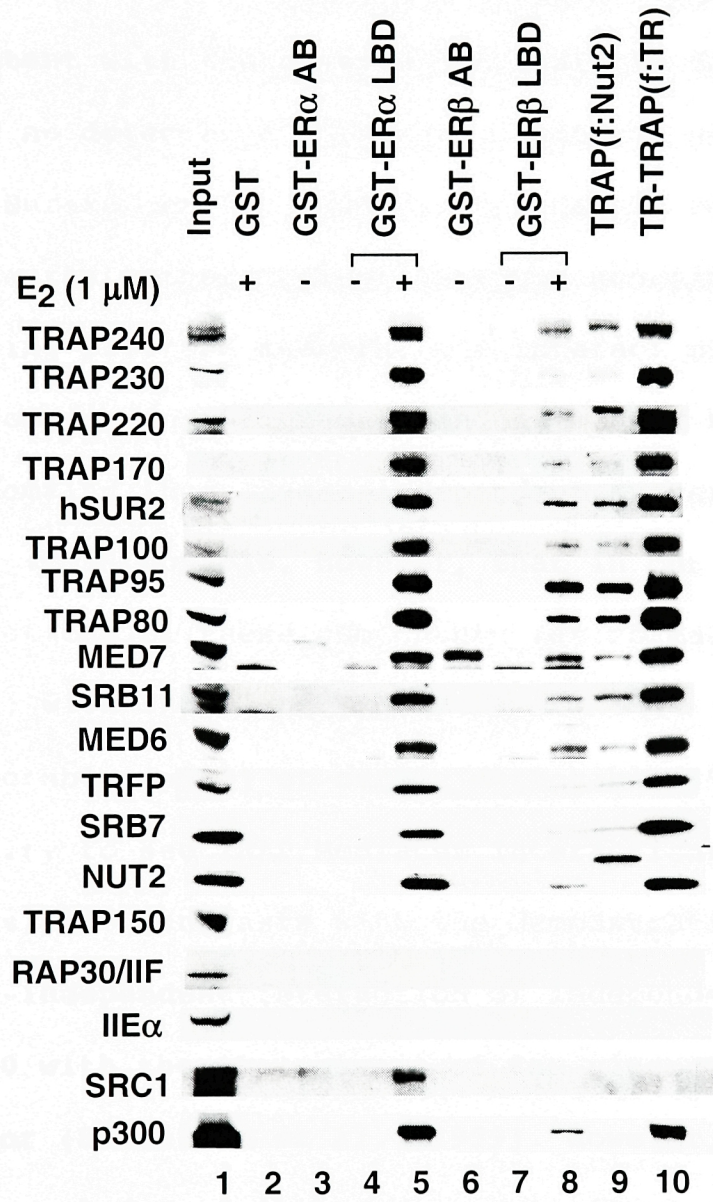
4.2. Results

4.2.1. Estrogen-dependent Interactions of TRAP/Mediator with ER LBDs in Nuclear Extracts

To extend the analyses of Fig. 3-2, proteins bound to ER LBDs in the presence and absence of E_2 were analyzed by western blot with antibodies to a more comprehensive set of TRAP/Mediator components. As shown in Fig. 4-1, and by comparison with purified TRAP (f:Nut2) and TR-TRAP (f:TR) complexes, all TRAP/Mediator components examined (other than TRAP150, which may not be a bona fide integral subunit) were found to associate with ERα and ERβ LBDs in an E_2-dependent manner. As equimolar amounts of immobilized fusion proteins were used for the binding assays, the results also confirmed a stronger interaction of TRAP/Mediator complex with the isolated ERα LBD than with the isolated ERβ LBD. The assays in Fig.4-1 further showed ligand-dependent interactions of SRC-1 and p300 with ER LBDs. As observed for TRAP/Mediator, these interactions are stronger for ERα than for ERβ. Consistent with the results of our previous analyses of TRAP/Mediator complexes (Gu et al., 1999; Malik et al., 2000), and indicative of specificity of factor binding to ER LBDs, no interactions of general

Fig. 4-1. E_2-dependent interactions of TRAP/Mediator with ERα and ERβ LBDs in nuclear extract. Immobilized GST (lane 2), GST-ERαAB (lane 3), GST-ERαLBD (lanes 4 and 5), GST-ERβAB (lane 6) and GST-ERβLBD (lanes 7 and 8) proteins were incubated with HeLa nuclear extract in the absence (-) or presence (+) of 1 μM E_2 and bound proteins were eluted and analyzed by SDS-PAGE and western blot (with antibodies to proteins indicated on the left) as described in Materials and Methods. One-tenth equivalent of the input nuclear extract is shown in lane 1. TRAP/Mediator and TR-TRAP complexes immunopurified from cells expressing f:Nut2 and f:TR respectively, are shown lanes 9 and 10. The band observed in lane 6 with MED7 antibody is a nonspecific band that cross-reacts with this antibody.

E_2-dependent interactions of TRAP/Mediator with ERα and ERβ LBDs in nuclear extract



transcription factors TFIIE (IIEα) and TFIIF (RAP30) were observed.

Also of note is the lack of any detectable binding of TRAP/Mediator components to GST-fused ERαAB and ERβAB regions that contain the AF-1 domains (Fig. 4-1). This is consistent with the observation that the isolated TRAP220 showed no detectable interaction with ER AF-1 but with ER AF-2 (Burakov et al., 2000). This result contrasts with reports that other nuclear receptor coactivators, including p160/SRC members, can interact not only with AF-2 domains in a ligand-dependent manner but also with AF-1 domains in a ligand-independent manner (Webb et al., 1998). It is notable, however, that in our assays interactions of these components (at normal nuclear levels) with ERαAB and ERβ AB domains were either undetectable (p300) or barely detectable (SRC-1). The inability to see TRAP/Mediator interactions with AB domains also contrasts with the demonstration of a ligand-independent interaction of independently expressed TRAP170 with the AF-1 domain of the glucocorticoid receptor (Hittelman et al., 1999). However, these results do not exclude possible modulatory effects of AB and associated AF-1 domains on interactions of TRAP/Mediator with LBD and associated AF-2 domains.

4.2.2. Direct Interactions of Purified TRAP/Mediator complex with ER LBDs

The studies described above have clearly shown ligand-dependent interactions of TRAP/Mediator with ER LBDs but, since they utilized nuclear extracts as a source of TRAP/Mediator, did not establish whether other factors were essential for these interactions. To further investigate this question, I employed a highly purified TRAP/Mediator complex immunopurified from cells expressing a FLAG-tagged SRB10/CDK8 subunit (Gu et al., 1999). As shown in Fig. 4-2, this complex showed an E_2-dependent interaction with the ERα LBD and an E_2-enhanced interaction with the ERβ LBD. Overall interactions in the presence of E_2 were again stronger for ERα than for ERβ, as in the analyses with nuclear extracts (above). Although I do not know the basis for the decreased ligand-dependency of the ERβ LBD interaction with purified TRAP/Mediator, relative to TRAP/Mediator in nuclear extracts (above), this may reflect the absence of negative constraints (interacting factors) that increase the ligand-dependency of interactions in nuclear extracts. This observation is also consistent with the observation of ligand-dependent interactions of ERβ with

Fig. 4-2. Direct interactions of purified TRAP/Mediator with LBDs of ERα and ERβ. Immobilized GST (lanes 6 and 7), GST-ERα LBD (lanes 2 and 3) and GST-ERβ LBD (lanes 4 and 5) were incubated with immunopurified TRAP/Mediator complex from cells expressing f:CDK8/SRB10 in the absence (-) or presence (+) of 1 μM E_2. After extensive washing, bound proteins were eluted and analyzed by SDS-PAGE and western blot (with antibodies to proteins indicated on the left) as described in Materials and Methods. One-tenth equivalent of the input TRAP/Mediator preparation was analyzed in lane 1.

Direct interactions of purified TRAP/Mediator with LBDs of ERα and ERβ

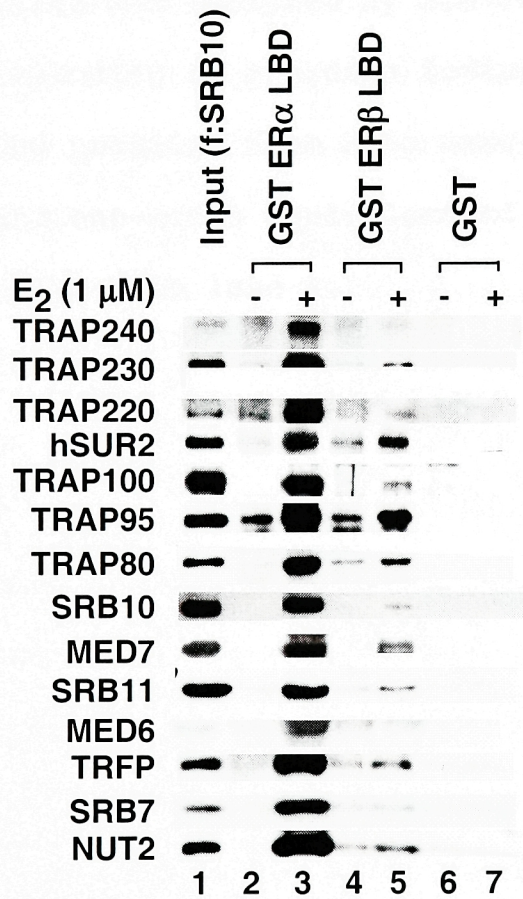


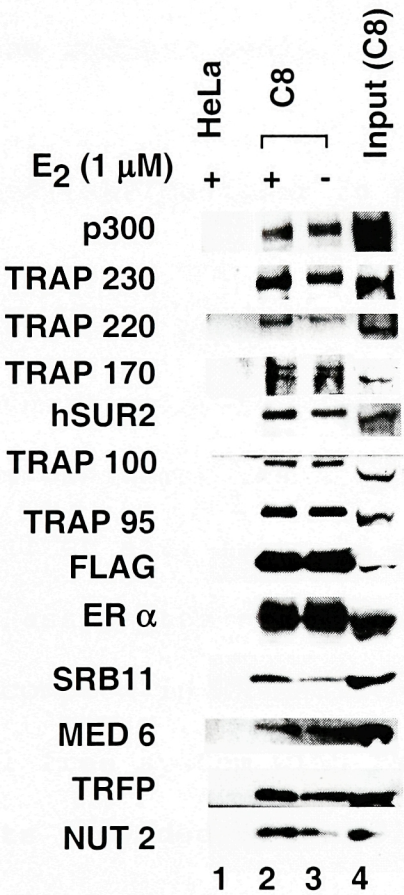
Fig. 4-3. Intracellular association of TRAP/Mediator with ERαΔAB. C8 cells that express FLAG-tagged ERα lacking the AB domain (f:hERαΔAB) were maintained in DME-PO_4 media supplemented with 10% calf serum. f:ERαΔAB and associated proteins were affinity-purified from C8-derived nuclear extract on M2 agarose. Proteins bound in the presence (+) or absence (-) of 1 μM E_2 during purification were eluted with FLAG peptide and analyzed by SDS-PAGE and western blot with antibodies to proteins indicated on the left. M2 agarose-bound proteins from HeLa extract were analyzed in lane 1 and one-tenth equivalent of C8 nuclear extract input was analyzed in lane 4.

a TRAP220 fragment in gel shift assays (Warnmark et al., 2001).

4.2.3. Intracellular Association of TRAP/Mediator with ERαΔAB

To provide evidence for a physiological association of ER with TRAP/Mediator, as previously demonstrated for TR (Fondell et al., 1996), I isolated FLAG-tagged ERαΔAB (f:hERαΔAB) and associated proteins through affinity-purification from C8-derived nuclear extracts on M2 agarose beads. The C8 cell line, established by Chao-Xing Yuan, is a HeLa-derived cell line that stably expresses a FLAG-tagged ERα lacking the AB domain (ERαΔAB). A western blot of the bound and eluted proteins revealed the presence of FLAG-tagged ERαΔAB (scored by both ERα and FLAG antibodies) as well as all TRAP/Mediator components that were analyzed (Fig. 4-3). An intracellular association of ERαΔAB with p300 was also detected. None of these proteins were bound to M2 agarose when a control extract from HeLa cells was applied. These results thus indicate an intracellular association of the entire TRAP/Mediator complex with ER, as originally described for TR.

Intracellular association of TRAP/Mediator with ERαΔAB



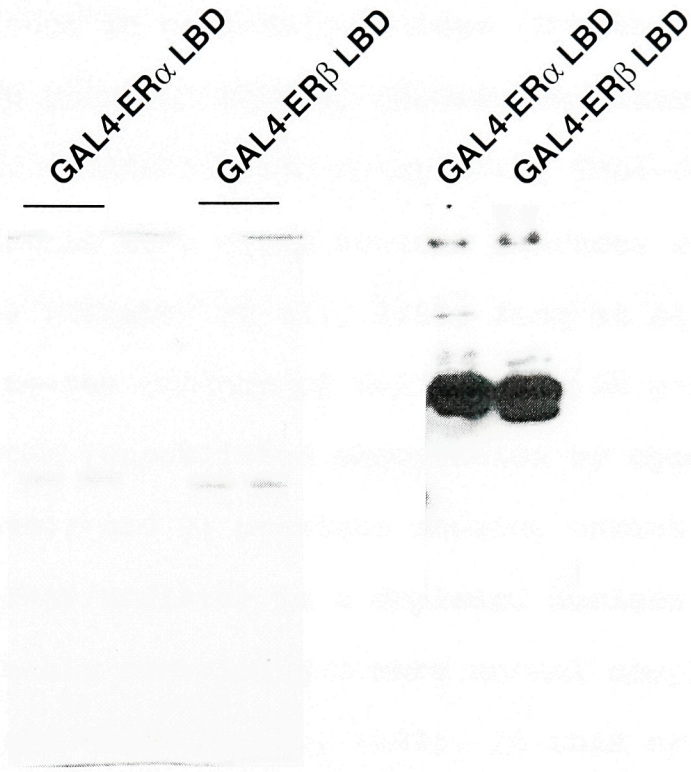
In the current analysis the probable E_2-dependence of this association could not be assessed, as the C8 cells lost receptors and exhibited poor viability in steroid-depleted media; and while purification of the ERαΔAB-containing complex in the presence and absence of E_2 yielded comparable results (Fig. 4-3, lane 2 vs. lane 3), this likely reflects prior occupancy and stability of the ER ligand binding pocket by estrogen or estrogen-like compounds in the culture media.

4.2.4. Roles for TRAP/Mediator In ER Function In nuclear extract.

TRAP/Mediator has been shown to enhance the transcription activity of several activators, including nuclear receptors, from DNA templates in purified cell free systems (Fondell et al., 1996; Gu et al., 1999; Ito et al., 2000). To assess the role of the complete TRAP/Mediator complex in ER-mediated transcription, I employed a cell free system with TRAP/Mediator depleted nuclear extracts provided by Hwa Jin Baek (Baek et al., 2002), combinations of baculovirus-expressed and purified GAL4-ER LBDs consisting of the DNA binding domain of the yeast activator GAL4 fused to the ER LBD (Fig.4-4) and affinity purified TRAP/Mediator from f:Nut2-derived

Fig. 4-4. Recombinant FLAG-tagged GAL4-ER LBD proteins. Affinity purified GAL4-ER LBD proteins were analyzed by SDS-PAGE and by either Coomassie Brilliant Blue R-250 staining (left panel) or western blot with anti-FLAG antibody (right panel).

Recombinant GAL4-ER LBD proteins

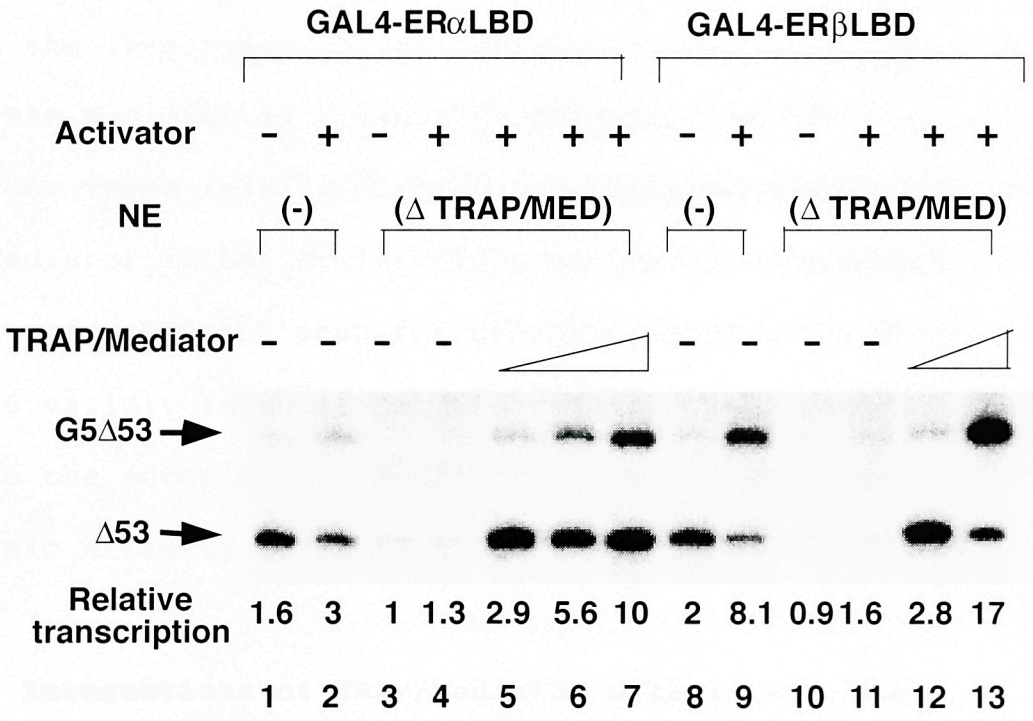


coomasie staining western blot

nuclear extract and a DNA template containing five copies of an GAL4 binding sites upstream of the adenovirus major late core promoter (Kundu et al., 2000). The rationale for this approach was: 1) a previous study showing that GAL4-fused ERα LBD activates transcription in a ligand-dependent manner in cell-based assays (Webster et al., 1988); 2) the previous studies showing the function of ER in an in vitro transcription assay using GAL4-fused ERα LBD in conjuntion with crude nuclear extracts from naked DNA templates (Webster et al., 1988; Jacq et al., 1994) in contrast to the failure of full-length ER proteins in the same system (unpublished observation by myself and Chao-Xing Yuan); and 3) previous studies assessing the activity of TRAP/Mediator in a depleted nuclear extract system presumably containing a more normal complement of nuclear factors (Baek et al., 2002). In this assay (Fig. 4-5), GAL4-ERα LBD (lanes 1 and 2) activated transcription weakly (circa 2-fold) and GAL4-ERβ LBD (lanes 8 and 9) activated circa 4-fold in untreated nuclear extracts. By contrast, in the TRAP/Mediator-depleted nuclear extract (ΔTRAP/MED) the absolute levels of activated transcription were completely reduced to the basal level (lanes 3 and 4, and lanes 10 and 11). Also of note, the levels of basal (activator-independent)

Fig. 4-5. Requirement of TRAP/ Mediator for transcriptional activity of GAL4-ER LBDs in nuclear extract. Transcription activation by GAL4-ER LBDs is suppressed in the α-TRAP25 antibody-depleted nuclear extract. In vitro transcription reactions contained 20 μg each nuclear extract (lanes 1,2,8,9: untreated; lanes 3-7,10-13: α-TRAP25 antibody-depleted) and 50 ng each of pΔ53 and pG5Δ53 templates, respectively. The transcription reactions in lanes 1-7 and lanes 8-13 additionally contained 10 ng of recombinant GAL4-ERα LBD and GAL4-ERβ LBD respectively. Increasing amount of purified TRAP/ Mediator (lanes 5-7 and lanes 12,13) were added to α-TRAP25 antibody-depleted nuclear extract in in vitro transcription reactions. As determined by immunoblotting, the amount of TRAP/ Mediator added to the reactions corresponded to approximately 100 % (lanes 5,12) or 200 % (lanes 6,13) or 400 % (lane 7) of the of the TRAP/ Mediator concentration in untreated nuclear extract. Relative transcription levels, determined by phosphorimaging, are indicated.

Requirement of TRAP/Mediator for transcriptional activity of GAL4-ER LBDs in nuclear extract



transcription (lanes 1 vs.3 and lanes 8 vs.10) were decreased upon depletion of TRAP/Mediator as reported previously (Baek et al., 2002). Upon addition of increasing amounts of TRAP/Mediator to depleted extract, activated transcription was, in a dose-dependent manner, enhanced up to seven-fold in the case of GAL4-ERα LBD and up to twenty-fold in the case of GAL4-ERβ LBD. This indication that an excess amount of ectopic TRAP/Mediator can enhance activated transcription by GAL4-ER LBDs beyond the level seen in the control extract suggests that TRAP/Mediator is a limiting cofactor for ER function. These results clearly demonstrated a role for TRAP/Mediator in ER-LBD function, as well as a greater activity for ERβ LBD than for ERα LBD (lanes 2 vs. 9 and lanes 6 vs.13). In addition, the relative responses of ER LBDs to the ectopic TRAP/Mediator correlate with the intrinsic affinity of ER LBDs for TRAP/Mediator.

4.2.5. Interactions of TRAP/Mediator with Intact ERs.

The above results have established E_2-dependent interactions of TRAP/Mediator with isolated ER LBDs, but these interactions can be considered physiologically relevant only if they are demonstrable in the context of natural full-length receptors. In an analysis of this

question, full-length FLAG-tagged ERs were expressed via baculovirus vectors, affinity purified and immobilized on M2 agarose beads (Fig.4-6). Nuclear extracts were applied to beads containing immobilized receptors, and after washing bound proteins were eluted and analyzed by SDS-PAGE and western blotting with antibodies to representative TRAP/Mediator components. As indicated in Fig. 4-7, TRAP/Mediator bound to both ERα and ERβ in an E_2-dependent manner. However, in contrast to the results with ER-LBDs, TRAP/Mediator showed stronger interactions with ERβ than with ERα. Although not directly comparable, these results are nonetheless consistent with a prior report that isolated TRAP220 interacts more strongly with intact ERβ than with ERα (Warnmark et al., 2001). Given that the assays for TRAP/Mediator interactions with intact ERs (Fig.4-7) versus ER LBDs (Fig. 4-1) were performed under comparable conditions, these results suggest that the ERα and ERβ AB domains differentially modulate interactions of TRAP/Mediator with corresponding LBDs.

To investigate the possibility that additional nuclear proteins might have facilitated intact ER-TRAP/Mediator interactions in these assays, the binding of ^{35}S-labelled full length ERβ to purified and

Fig. 4-6. Recombinant FLAG-tagged ER proteins. Affinity purified ERs were analyzed by SDS-PAGE and by either Coomassie Brilliant Blue R-250 staining (lanes 1 and 2) or western blot with corresponding antibodies (lanes 3 and 4).

Recombinant ERα and ERβ

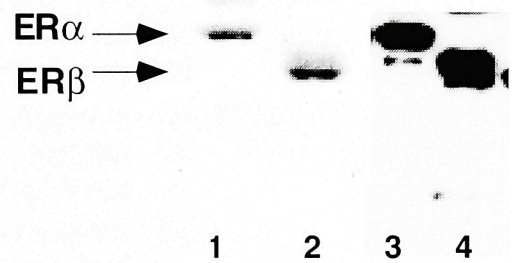
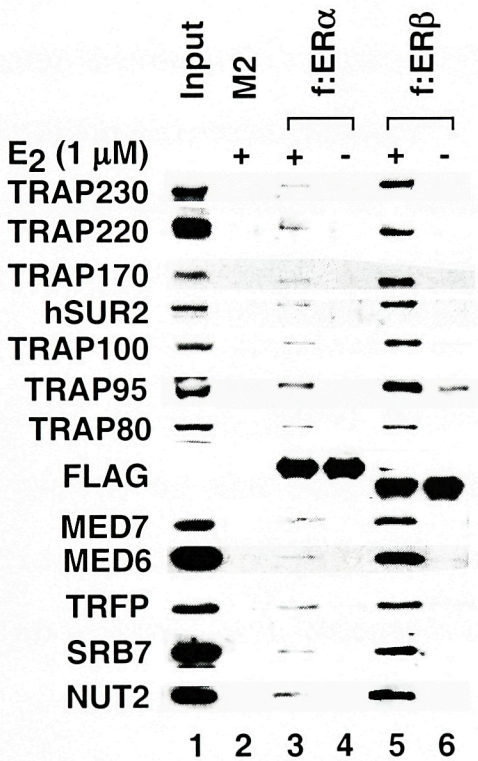


Fig. 4-7. E_2-dependent interactions between TRAP/Mediator and ERs in HeLa nuclear extract. M2 agarose-immobilized FLAG-ERα (lanes 3 and 4) and FLAG-ERβ (lanes 5 and 6) were incubated with HeLa nuclear extracts in the absence (-) or presence (+) of 1 μM E_2 and bound proteins were eluted with FLAG peptide and analyzed by SDS-PAGE and western blot (with antibodies to proteins indicated in the left) as described in Materials and Methods. As a control, HeLa nuclear extract proteins bound to M2 agarose alone were analyzed in lane 2. One-tenth of input nuclear extract was analyzed in lane 1.

E_2-dependent interactions between TRAP/Mediator and full-length ERs in HeLa nuclear extract



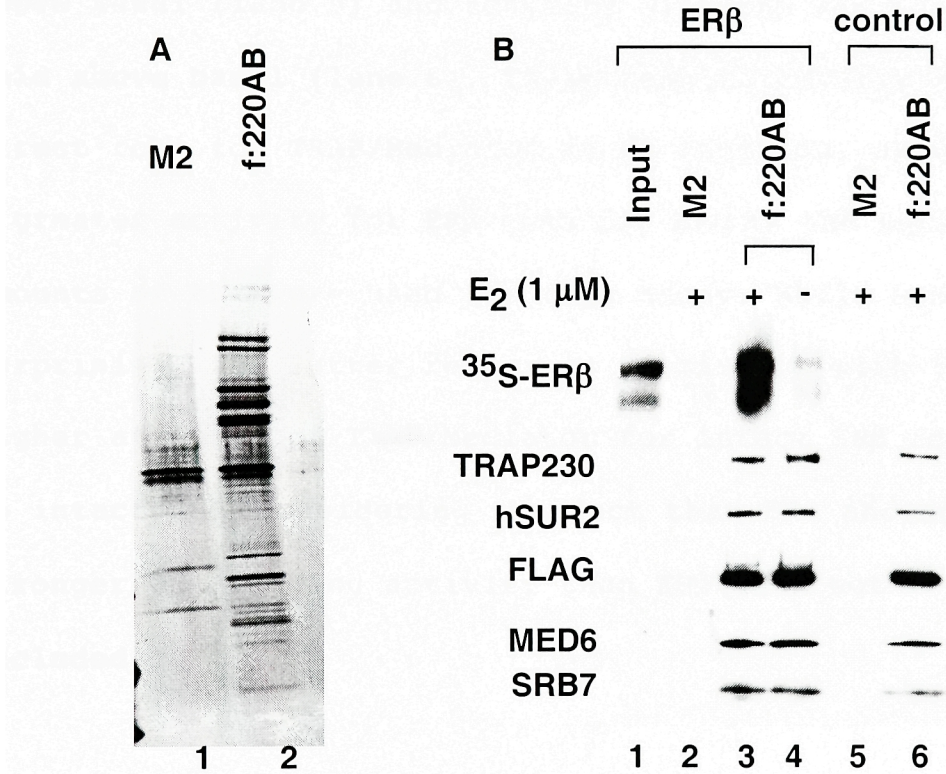
immobilized TRAP/Mediator (Fig.4-8A) was analyzed. For technical reasons relating to the presence of FLAG tags both on recombinant ERs and on the purified TRAP complex, an analysis of TRAP/Mediator binding to M2 agarose-immobilized ERs was not possible. As shown in Fig. 4-8B, ^{35}S-labelled full length ERβ bound to purified TRAP/Mediator, thus indicating direct ER-TRAP/Mediator interactions.

4.2.6. TRAP/Mediator Directly Enhances ER Function In a Purified In Vitro Transcription Assay.

TRAP/Mediator has been shown to enhance the transcription activity of several activators, including nuclear receptors, from DNA templates in purified cell free systems (Fondell et al., 1996; Gu et al., 1999; Ito et al., 2000). The role of the complete TRAP/Mediator complex in ER-mediated transcription, was further assessed, in collaboration with Mohamed Guermah, in a cell free system reconstituted with highly purified general initiation factors and cofactors (TFIIB, TFIID, TFIIE, TFIIF, TFIIH, RNA polymerase II and PC4), combinations of baculovirus-expressed and purified receptors (Fig.4-6) and affinity purified TRAP/Mediator (Fig.4-8A lane 2), and a DNA template containing four

Fig. 4-8. E_2-dependent interactions between purified TRAP/Mediator and full-length ERβ. A. Purified f:TRAP220AB TRAP/Mediator complex. The complex was affinity purified from cells expressing a FLAG-tagged TRAP220 lacked the C-terminal domain (f:TRAP220AB) and analyzed by SDS-PAGE and silver staining (lane 2). Lane 1 shows HeLa nuclear extract proteins bound non-specifically to M2 agarose. B. Direct interactions between purified TRAP/Mediator complex and full-length ERβ. M2 agarose immobilized TRAP/Mediator (f:TRAP220AB) complex (lanes 3,4 and 6) and M2 agarose alone (lanes 2 and 5) were incubated with ^{35}S-labeled (in vitro translated) full length ERβ in the presence (lane 3) or absence (lane 4) of 1 μM E_2 or with control lysate (lanes 5 and 6). After washing, bound proteins were eluted with FLAG peptide and analyzed by autoradiography (upper panel) or by western blot with antibodies to the indicated components of TRAP/Mediator complex (lower panel). Lane 1 shows one-twentieth of the ^{35}S-labeled full length ERβ input.

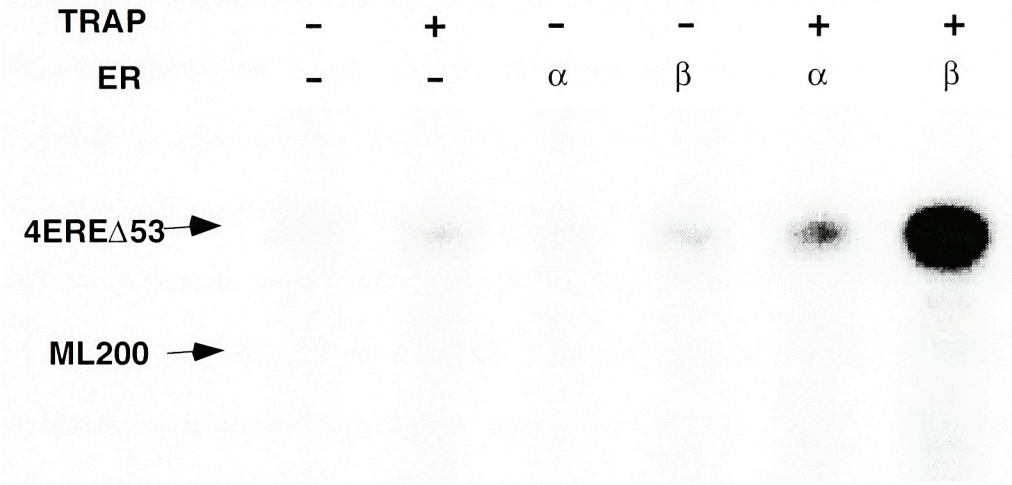
E_2-dependent interactions between purified TRAP/Mediator and full-length ERβ



copies of an estrogen response element (ERE) upstream of the adenovirus major late core promoter (Wu et al., 1999). In this assay (Fig. 4-9), basal activity (lane 1) was unaffected by ERα alone (lane 3) and weakly (circa 2-fold) enhanced by ERβ alone (lane 4) or by TRAP/Mediator alone (lane 2). In the presence of TRAP/Mediator, activity with ERα was about five-fold above basal (lane 5) and activity with ERβ was about 20-fold above basal (lane 6). These results clearly show a direct role for TRAP/Mediator in ER function, as well as a greater activity for ERβ than for ERα as the equimoloar amounts of ERs were used for this assay. While somewhat surprising, the latter result is consistent with the higher affinity of TRAP/Mediator for intact ERβ relative to intact ERα considering the fact that ERα showed the stronger DNA binding activity than ERβ (data not included).

Fig. 4-9. TRAP/Mediator complex directly mediates ER function in vitro. Reactions contained purified general transcription factors and cofactors, purified TRAP/Mediator complex and ER proteins (20 nM) as indicated, and both 4EREΔ53 G-less reporter and ML200 G-less control templates. TRAP/Mediator was purified from the cell line expressing f:TRAP220AB (Fig. 4-8A lane 2).

TRAP/Mediator complex directly mediates ER function in vitro



Chapter 5

Role of TRAP220 subunit in ER Function

5.1. Introduction

The significance of TRAP220 subunit in Nuclear receptor function has been well established by studies showing: 1) original isolations through its direct interaction with TR (Lee et al., 1995) and PPARγ (Zhu et al., 1997) in yeast two-hybrid assays; 2) ligand-dependent interactions of TRAP220 with a number of other nuclear receptors, further suggesting a broader role for TRAP220 through TRAP/Mediator in nuclear receptor function (Yang et al., 2000; Rachez et al., 1999; Yuan et al., 1998 ; Zhu et al., 1997); 3) defective cellular functions of various receptors in response to dominant negative mutant form of TRAP220 (Yuan et al., 1998; Zhu et al., 1997); 4) significant physiological defects including receptor function upon deletion of TRAP220 (Ito et al., 2000; Shao et al., 2000; Zhu et al., 2000; Ge et al., 2002).

Despite the lack of any direct evidence for the role for the TRAP/Mediator complex in ER function, a number of studies suggested the potential involvement of TRAP220 subunit in ER function. Chao-Xing Yuan first observed a ligand-dependent interaction of intact TRAP220 with ERα although it was very weak compared to other receptors

(Yuan et al., 1998). Subsequent studies confirmed physical interactions of TRAP220 with ERα (Burakov et al., 2000; Warnmark et al., 2001; Zhu et al., 1999), demonstrated inhibitory effects of an ER-interacting fragment of TRAP220 (Burakov et al., 2000) and an anti-TRAP220 antibody on ERα function in transfected cells, and established the presence of TRAP220 on the promoters of endogenous estrogen-responsive genes (Shang et al., 2000). However, interpretation of these studies was complicated by (1) the stable association of TRAP220 with other TRAP/Mediator components that may mediate (via different activators not ER) TRAP/Mediator recruitment and function; (2) the failure to analyze suitable control genes and other cofactors for broader effects of agents designed to block TRAP220 functions; and (3) some discrepancy regarding the ability of TRAP220 to interact with ERα vs. ERβ (mentioned in Kang et al., 2002).

Not surprisingly, TRAP220 contains two LXXLL motifs that are found in most of nuclear receptor cofactors and responsible for the interaction with nuclear receptors. The LXXLL motif has been suggested to play roles in determining specificity from the observation that different LXXLL motif is required for the interaction with specific nuclear receptors. Consistent with this

idea, the second (closer to C-terminal) LXXLL motif (NR box2) in TRAP220/DRIP205 was essential for the interaction with VDR and TR, whereas the first (more N-terminal) motif (NR box1) was dispensible (Yuan et al., 1998; Rachez et al., 2000).

Here I characterized a role for TRAP220 in ER function. I showed that TRAP220 is an essential anchor for a strong interaction between ER and TRAP/Mediator. I further demonstrated that both NR boxes within TRAP220 are important for mediating an interaction between ER and the TRAP/Mediator complex. I also describe my preliminary studies on the function of TRAP220 and its NR boxes in ER-dependent transcription.

5.2. Results

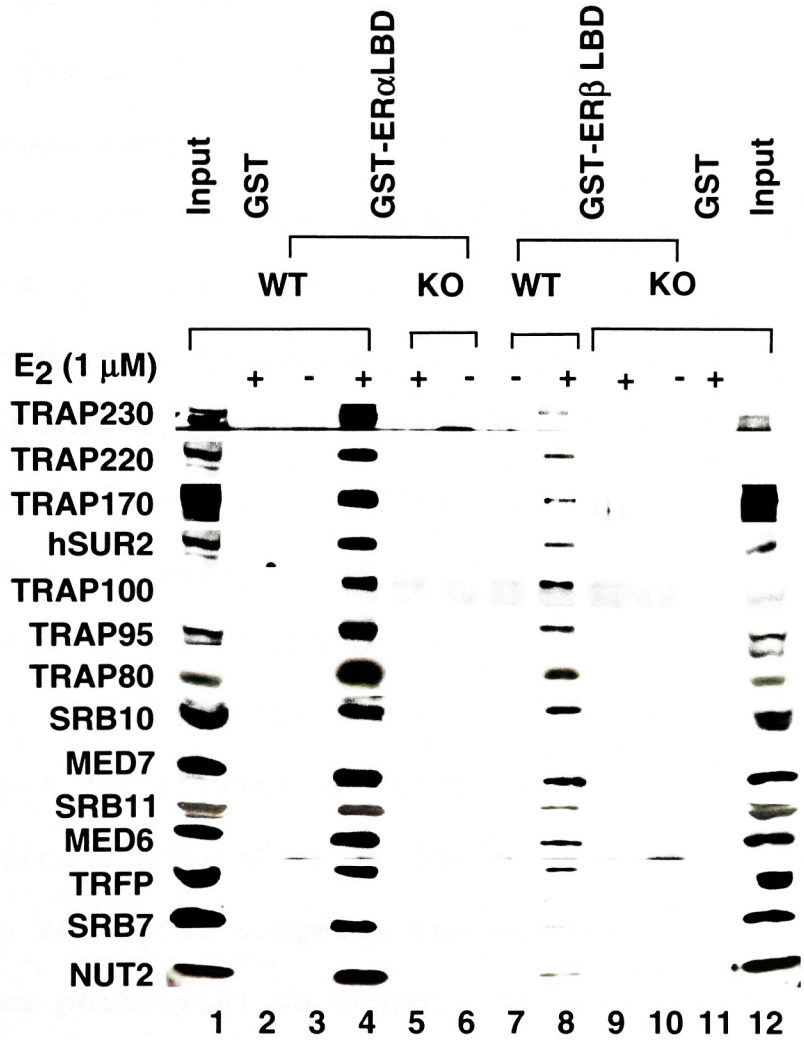
5.2.1. TRAP220-dependent Interactions of TRAP/Mediator with ER LBDs in Nuclear Extracts.

The demonstration of ligand-dependent interactions of ERα and ERβ with isolated TRAP220 (Burakov et al., 2000; Warnmark et al., 2001; Yuan et al., 1998; Zhu et al., 1999) and the observation that TRAP220 is a main subunit for strong ligand-dependent interactions of the TRAP/Mediator complex with nuclear receptors such as TR

and VDR (Yuan et al., 1998; Rachez et al., 1999), has led to speculation that TRAP220 may anchor ERs to the TRAP/Mediator complex. To address this issue, I employed GST pull down assays with nuclear extracts from MEFs derived from wild type and TRAP220-/- mice (Ito et al., 2000). This approach is technically simpler than other alternative approaches such as a Far Western analysis employed for TR (Yuan et al., 1998). The availability of reagent further facilitated this assay. Furthermore, this assay can determine the role for TRAP220 in the observed ER-TRAP/Mediator interaction. As shown in Fig. 3-4 and Fig.5-1, TRAP/Mediator components from control MEFs bound in an E_2-dependent manner to GST-fused ER LBDs whereas TRAP/Mediator components from TRAP220-/- MEFs did not. Other studies have shown that TRAP220-/- cells contain a residual TRAP complex lacking only TRAP220 and that this complex interacts normally with other activators such as VP16 (Chao-Xing Yuan and Sohail Malik unpublished observation). These results firmly establish an essential role for TRAP220 in strongly anchoring TRAP/Mediator to ERα and ERβ and again show that TRAP/Mediator has a stronger affinity for the isolated ERα LBD than for the isolated ERβ LBD in the context of other nuclear proteins.

Fig. 5-1. TRAP220-dependent interactions of TRAP/Mediator with ER LBDs in nuclear extract. Immobilized GST (lanes 2 and 11), GST-ERα LBD (lanes 3-6) and GST-ERβ LBD (lanes 7-10) were incubated with the nuclear extracts from wild type (WT) (lanes 2,3,4,7 and 8) and TRAP220$^{-/-}$(KO) MEFs (lanes 5,6,9,10 and 11) in the absence (-) or presence (+) of 1 μM E_2 and bound proteins were eluted and analyzed by SDS-PAGE and western blot (with antibodies to proteins indicated on the left) as detailed in Materials and Methods. One-tenth equivalents of input nuclear extracts were analyzed in lanes 1 (WT) and 12 (KO).

TRAP220-dependent interactions of TRAP/Mediator with ER LBDs in nuclear extract



5.2.2. Effect of TRAP220 on ER-driven transcriptional activation in TRAP220$^{-/-}$ MEFs.

The essential requirement of TRAP220 for the ER-TRAP/Mediator interaction raised the possibility that loss of TRAP220 could result in the failure to support ER-activated transcription. To examine this possibility, I carried out transfection assays with WT and TRAP220-/- MEFs employed in the previous pull down assays. MEFs of each genotype were transfected with an ER expression vector and a reporter containing three ERE sites and core promoter. A strong E_2 response (up to 80-fold induction) was observed in wild type cells. The response was attenuated only modestly (circa 2-fold), but very reproducibly, in TRAP220-/- MEFs (Fig. 5-2). Significantly, however, transcription in TRAP220-/- MEFs was restored to the wild-type level by simultaneous expression of ectopic TRAP220 (Fig. 5-3). While TRAP220 is essential for optimal ER function in response to E_2, the significant activation of ER-driven transcription in TRAP220-/- MEFs that suggests the existence of an alternative pathway(s) to support ER function or lack of proper restraint, which TRAP/Mediator is required to overcome (see discussion).

Fig. 5-2. Defect in ER-mediated transcription in TRAP220$^{-/-}$ MEFs. The MEFs of each genotype were transfected with indicated amounts of human ER, 3ERE-luciferase reporter, and control luciferase pRL-CMV. Cells were cultured in the absence (-) or presence (+) of the ligand, E_2 (10^{-8} M), and dual luciferase activities were measured 36 hr after transfection. Values (means ± SD of a representative experiment performed in duplicate) are plotted as a fold.

Defective ER-driven transcription in TRAP220-/- MEFs

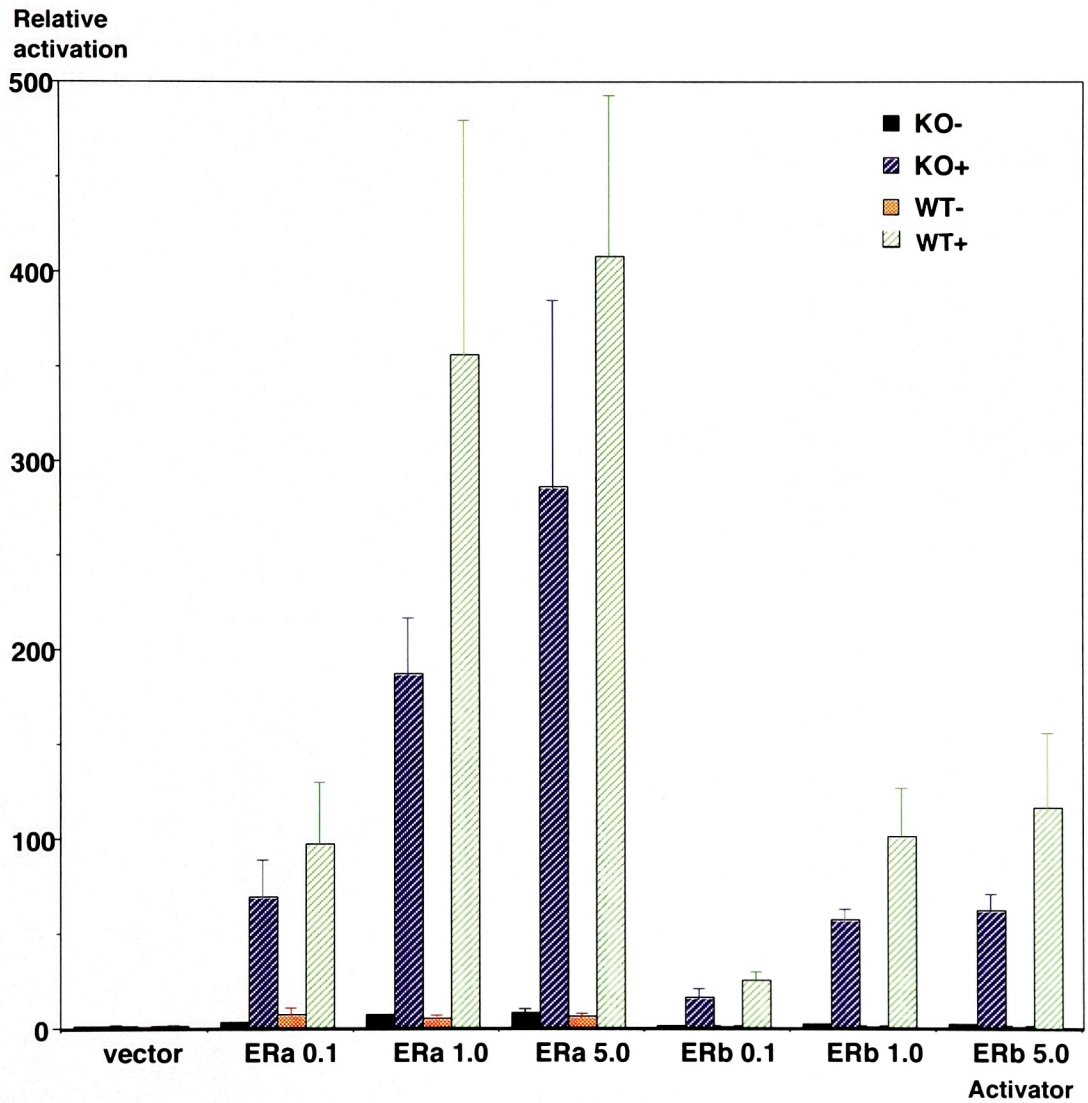
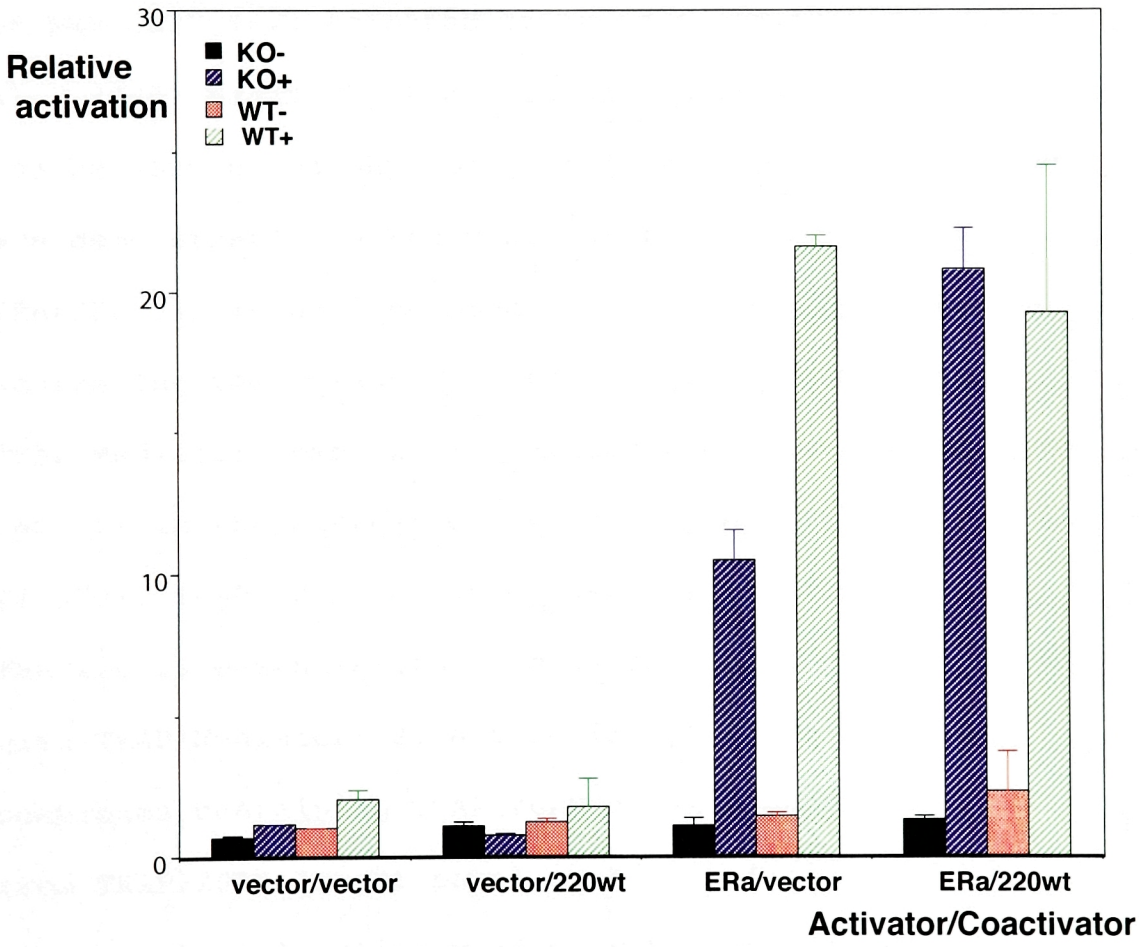


Fig. 5-3. Complementation of defective ER-driven transcription in TRAP220-/- MEFs by exogenous TRAP220. The MEFs of each genotype were transfected with human ER, TRAP220, 3ERE-luciferase reporter, and control luciferase pRL-CMV. Cells were cultured in the absence (-) or presence (+) of the ligand, E_2 (10 nM), and dual luciferase activities were measured 36 hr after transfection. Values (means ± SD of a representative experiment performed in duplicate) are plotted as a fold.

Complementation of defective ER-driven transcription in TRAP220-/- MEFs by exogenousTRAP220



5.2.3. NR box-dependent Interactions of isolated TRAP220 with ER LBDs

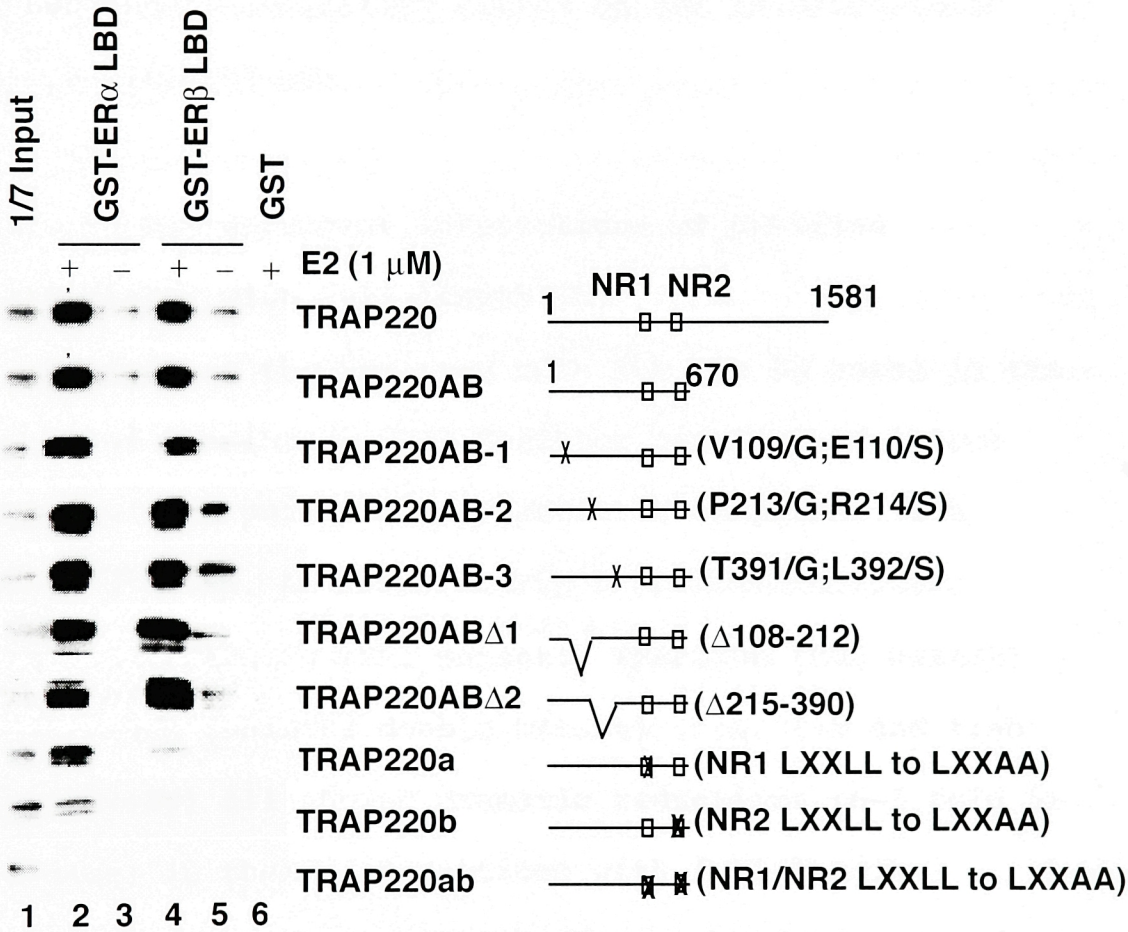
The differential requirement of each NR box in TRAP220 for the interaction with ERs had been suggested by the previous observations that deletion or mutation of NR1 in the fragment of TRAP220 (604-774) abolished or decreased interaction with ERα while deletion or mutation of NR2 (527-604) affected it to less extent (Burakov et al., 2000; Warnmark et al., 2001). However, these results can be considered physiologically relevant only if they are demonstrable in the context of natural full length TRAP220 and intact TRAP/Mediator complex. Prior to addressing this issue in the context of intact TRAP/Mediator complex, I examined the relevance of these results in the context of the N-terminal part of TRAP220 (1-670), TRAP220 AB, on the basis of the following: 1) TRAP220 is essential for demonstrable interaction of ER with TRAP/Mediator; 2) availability of TRAP/Mediator complexes containing TRAP220AB or each mutant derived from TRAP220AB (right panel in Fig. 5-4); and 3) the transcriptional activity of TRAP/Mediator complex containing TRAP220AB to support TR function is equivalent to that of TRAP/Mediator containing full-length TRAP220 (unpublished observation by Mohamed Guermah and Chao-Xing

Yuan). I employed GST pull down assays using GST-ER LBDs with ^{35}S-labeled proteins expressed from the constructs (right panel in Fig. 5-4) harboring deletion mutants or point mutants of TRAP220AB (provided by Chao-Xing Yuan). Apart from TRAP220 NR mutants, I used various TRAP220AB mutants in order to examine the possible involvement of other part(s) of TRAP220 in the interaction between ER and TRAP220 because the previous experiments used only fragments of TRAP220 (Burakov et al., 2000). As shown in Fig. 5-4, most of the mutants exhibited strong interactions with GST-ER LBDs comparable to WT full-length and AB fragment. In contrast, point mutations in either NR box significantly diminished the TRAP220 interaction with ER LBD. Morevore, in contrast to the results of previous reports (Burakov et al., 2000; Warnmark et al., 2001), mutation in NR2 exhibited a more severe effect than in NR1. This might be due to the differences in assay conditions and/or TRAP220 constructs that were used. In addition, the interaction between GST-ER LBDs and TRAP220AB with the mutations in both NR boxes was undetectable.

Also of note, ERα LBD and ERβ LBD seemed to bind to isolated TRAP220 similarly in this assay. This is consistent with the earlier study by Burakov et al but is

Fig. 5-4. NR box-dependent interactions of TRAP220 with ER LBDs. Immobilized GST (lanes 6), GST-ERα LBD (lanes 2 and 3) and GST-ERβ LBD (lanes 4 and 5) were incubated with ^{35}S-labeled (in vitro translated) TRAP220 proteins in the presence (lane 2 and 4) or absence (lane 3 and 5) of 1 μM E_2 and bound proteins were eluted and analyzed by SDS-PAGE followed by autoradiography as detailed in Materials and Methods. Lane 1 shows one-seventh of the ^{35}S-labeled TRAP220 input.

E2-dependent Interaction between TRAP220 mutants and GST-ER LBDs



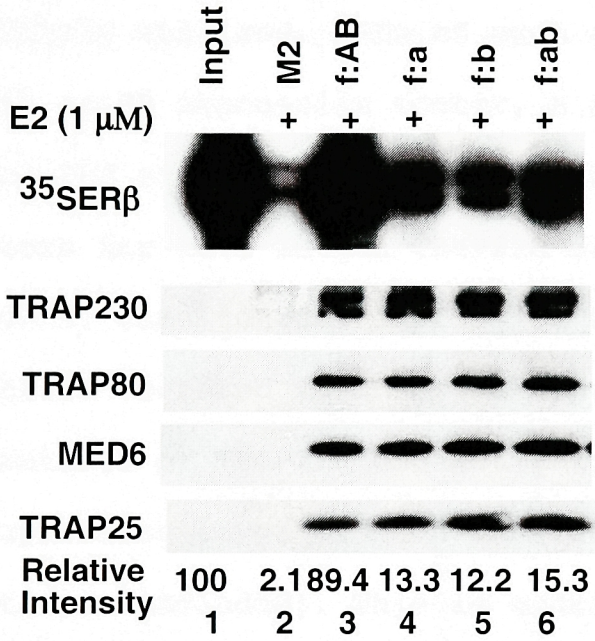
contrast to the study by Warnmark et al. More importantly, this result seems to be contrast to the result of many assays with nuclear extract and purified TRAP/Mediator complex. Although we do not know the reason yet this could be due to stimulatory effect of other Mediator components on the interaction of TRAP220 with ERα LBD and/or inhibitory effect on the interaction of TRAP220 with ERβ LBD.

5.2.4. NR box-dependent Interactions of purified TRAP/Mediator with full-length ERβ

To analyze the observed role for the NR boxes in the context of the intact TRAP/Mediator complex and intact ER, I employed purified TRAP/Mediator complexes with full-length ERβ. As shown in Fig 5-5, TRAP/Mediators containing TRAP220a (NR1 mutant), TRAP220b (NR2 mutant) or TRAP220ab (NR1/NR2 double mutant) (f:a, f:b and f:ab respectively) all showed dramatic reductions (6-8 fold in each case) in their interactions with full-length ERβ, compared to that of TRAP/Mediator complex containing TRAP220AB (f:AB). This indicated that the point mutations in either NR box significantly affect the interaction between intact TRAP/Mediator and ERβ full-length and suggests that both NR boxes are important for the

Fig. 5-5. TRAP220 NR box-dependent interaction between purified TRAP/Mediator complex and full-length ERβ. M2 agarose immobilized TRAP/Mediator (f:TRAP220AB, f:TRAP220a, f:TRAP220b and f:TRAP220ab) complexes (lanes 3,4, 5 and 6) and M2 agarose alone (lane 2) were incubated with ^{35}S-labeled (in vitro translated) full length ERβ in the presence (lanes 2-6) of 1 μM E_2. After washing, bound proteins were eluted with FLAG peptide and analyzed by autoradiography (top panel) or by western blot with antibodies to the indicated components of TRAP/Mediator complex (lower panels). Lane 1 shows one-seventh of the ^{35}S-labeled full length ERβ input.

TRAP220 NR box-dependent interaction between purified TRAP/Mediator and full-length ERβ



interaction between intact TRAP/Mediator and ERβ full-length.

5.2.5. Role for NR box of TRAP220 in ER-dependent transcription in TRAP220-/- MEFs

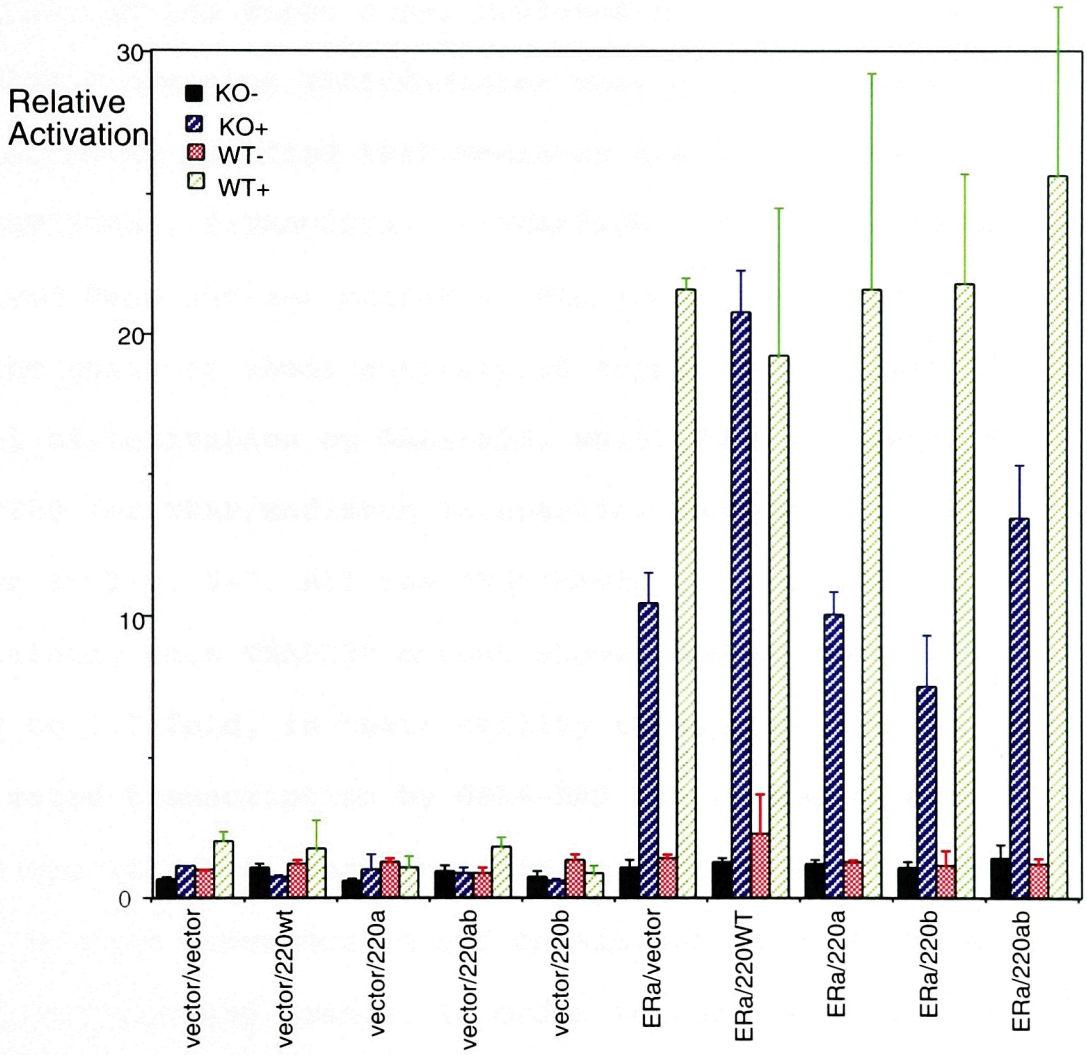
To assess the role of the TRAP220 NR boxes in ER dependent transcription I employed transfection assays with MEFs previously utilized. MEFs of each genotype were transfected with an ER expression vector, a reporter containing three ERE sites with core promoter and expression vectors for full length TRAP220 either wild-type or NR mutants. Simultaneous expression of ectopic full-length TRAP220 restored to the wild-type level while all the point mutants of TRAP220 did not (Fig. 5-6) although the expression level of each TRAP220 was comparable (data not included). This is consistent with the results from the interaction study.

5.2.6. Role for NR box of TRAP220 in ER-dependent transcription in cell free transcription assay

The observation of the modest defect in ER-driven transcription in TRAP220-/- MEFs raised the concern that this particular cell-based assay might not be able to fully recapitulate normal ER activation mechanisms and

Fig. 5-6. Role for TRAP 220 NR box in ER-driven transcription. The MEFs of each genotype were transfected with human ER, indicated TRAP220, 3ERE-luciferase reporter, and control luciferase pRL-CMV. Cells were cultured in the absence (-) or presence (+) of the ligand, E_2 (10^{-8} M), and dual luciferase activities were measured 36 hr after transfection. Values (means ± SD of a representative experiment performed in duplicate) are plotted as a fold.

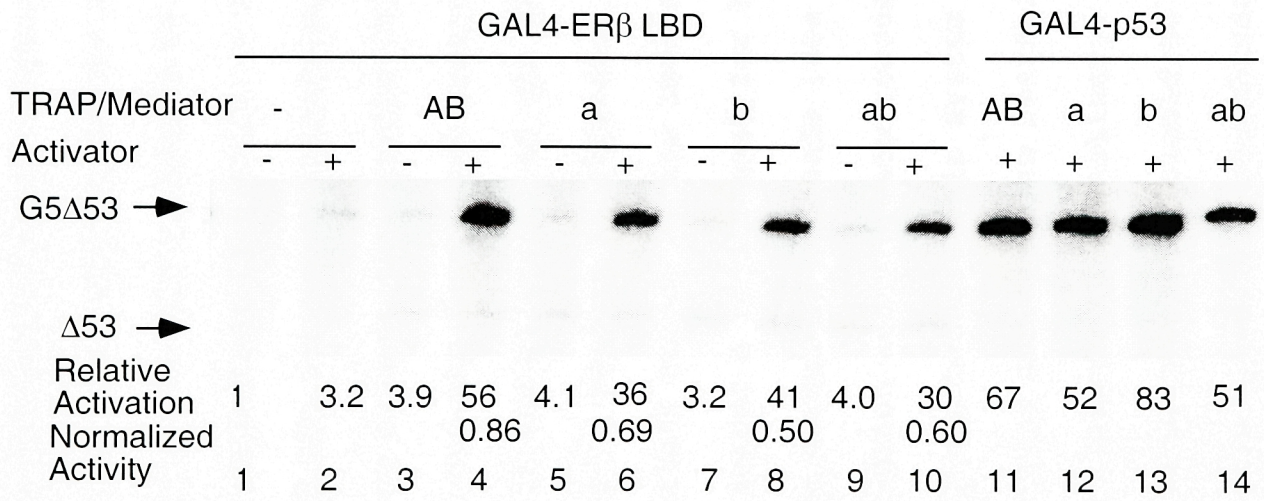
Role for TRAP220 NR box in ER-driven transcription



requirements for cofactors such as TRAP220. To extend the analysis, and given positve results in the previous observations (Fig.4-5 and Fig.4-9), I employed cell-free transcription assays with either TRAP/Mediator-depleted nuclear extract or a system reconstituted with purified factors. In the first case, depleted nuclear extracts lacking endogenous TRAP/Mediator were complemented with the affinity purified TRAP/Mediator complexes from f:TRAP220AB-, f:TRAP220a-, f:TRAP220b-, and f:TRAP220ab-derived HeLa nuclear extracts. The inputs were normalized on the basis of their activity to support equivalent level of activation by GAL4-p53, which does not require TRAP220 for TRAP/Mediator interaction and function. As shown in Fig. 5-7, all the TRAP/Mediator complexes containing each TRAP220 mutant showed modest reduction (1.2 to 1.7 fold) in their ability to support the activated transcription by GAL4-ERβ LBD in comparison with the TRAP/Mediator containing wild type TRAP220 AB. This is very reproducible and consistent with the results from transfection assays. In order to rule out the possible residual TRAP220 in the depleted nuclear extracts, I employed Namalwa nuclear extracts depleted TRAP/Mediator by anti-TRAP220 antibody. The composition of this assay system is essentially same as the system

Fig. 5-7. Role for TRAP220 NR box in ER-dependent transcription. In vitro transcription reactions contained 20 μg the α-TRAP25 antibody-depleted nuclear extract and 17.5 ng of pΔ53 and 35ng of pG5Δ53 templates. The transcription reactions in lanes 1-10 and lanes 11-14 additionally contained 10 ng of recombinant GAL4-ERβ LBD and GAL4-p53 respectively. Each purified TRAP/ Mediator complex (f:TRAP220AB in lanes 3,4, and 11; f:TRAP220a in lanes 5,6, and 12; f:TRAP220b in lanes 7,8, and 13;f:TRAP220ab in lanes 9,10 and 14) were added to α-TRAP25 antibody-depleted nuclear extract in transcription reactions. Relative transcription levels, determined by phosphorimaging, are indicated.

Role for TRAP220 NR box in transcriptional activity of GAL4-ERβ LBD in nuclear extract

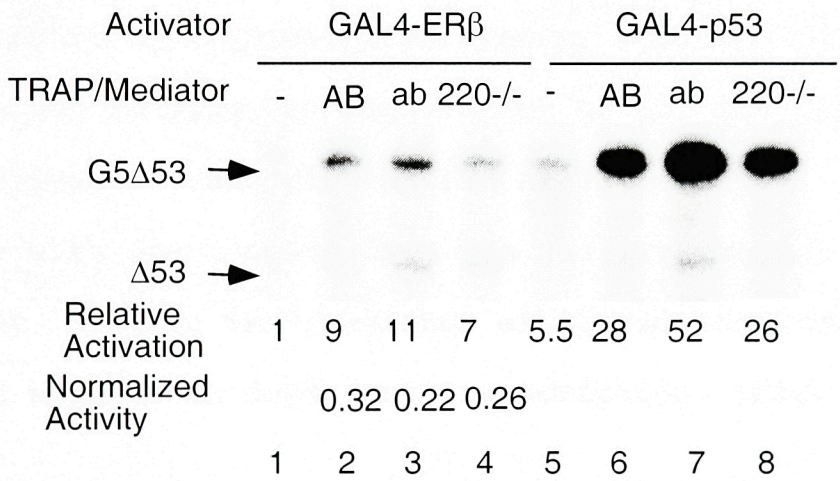


shown in Fig. 5-7. In this assay (Fig. 5-8), the level of activated transcription by GAL4-ERβ LBD was enhanced up to about 9 fold upon addition of TRAP/Mediator containing TRAP220 AB (f:AB) similar to previous experiment (Fig. 4-5). TRAP/Mediator containing either double mutant TRAP220ab (f:ab) or no TRAP220 (provided by Sohail Malik) showed very modest reduction (1.5 fold) in their ability to support GAL4-ERβ LBD-activated transcription.

The discrepancy between the results from the interaction assays and the functional assays could be due to the following: 1) the existence of the alternative activation pathways to support ER dependent transcription such as other cofactors and/or 2) the lack of the constraints which impose a requirement for TRAP/Mediator to overcome such as chromatin blockade (see discussion). I employed transcription assay system reconstituted with highly purified factors because this system is so minimal that neither activation nor negative restraints present other than general transcription machinery. So if the first possibility is major then I should be able to see the difference in this sytem. The recombinant factors expressed in and purified from bacteria included TFIIB (one subunit [Fig. 5-9A, lane1]), TBP (lane 2), TFIIE (two subunits [lane 3]), TFIIF (two subunits [lane 4])

Fig. 5-8. Role for TRAP220 in transcriptional activity of GAL4-ERβ LBDs in nuclear extract. In vitro transcription reactions contained 20 μg the α-TRAP25 antibody-depleted nuclear extract and 17.5 ng of pΔ53 and 35ng of pG5Δ53 templates. The transcription reactions in lanes 1-4 and lanes 5-8 additionally contained 10 ng of recombinant GAL4-ERβ LBD and GAL4-p53 respectively. Each purified TRAP/ Mediator complexes (f:TRAP220AB in lanes 2 and 6; f:TRAP220ab in lanes 3 and 7; and f:TRAP220-/- in lanes 4 and 8) were added to α-TRAP25 antibody-depleted nuclear extract in transcription reactions. Relative transcription levels, determined by phosphorimaging, are indicated.

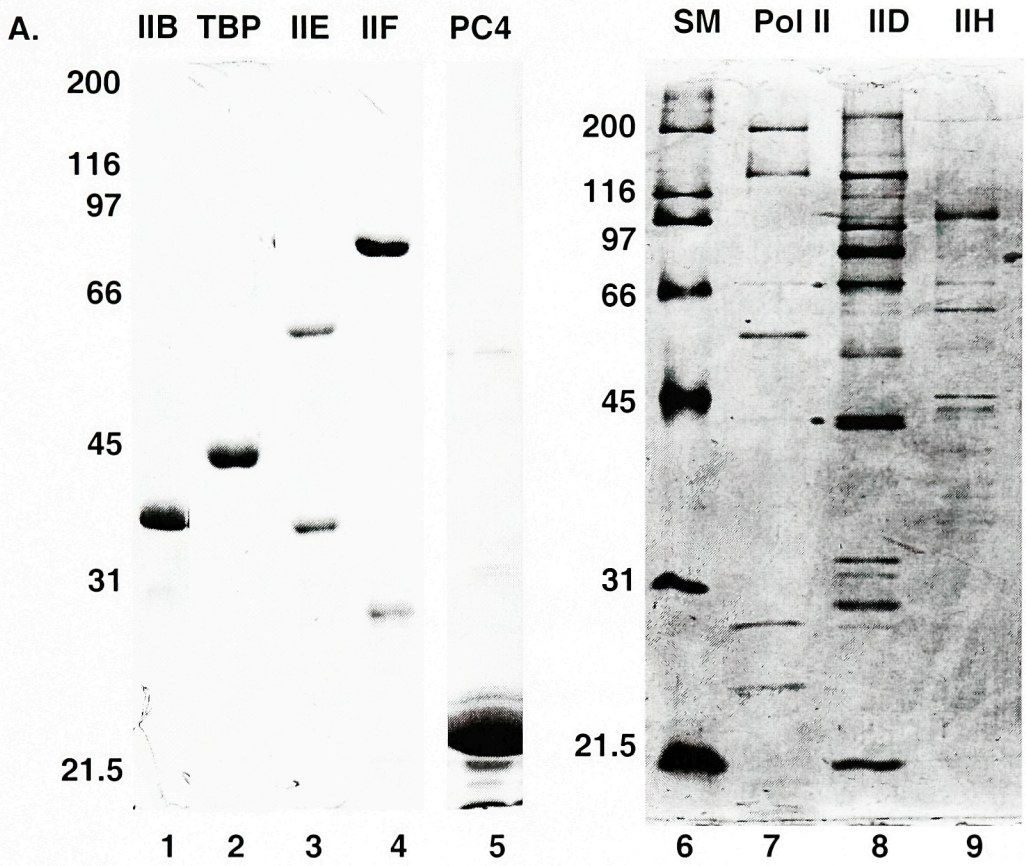
Effect of mutation or loss of TRAP220 in TRAP/Mediator on transcriptional activity of GAL4-ERβ LBD in Nuclear Extract



and PC4 (1 subunit [lane 5]). The multisubunit components purified from cell lines expressing FLAG-tagged subunits included f: Pol II (12 subunits [lane 7]), f:TFIID (~15 subunits [lane 8]) and f:TFIIH (9 subunits [lane 9]). Recombinant GAL4-p53 was used as an activator to establish the functionality of this particular assay system. As shown in Fig. 5-9B, activation (up to 19-fold) by GAL4-p53 was achieved above the basal (activator independent) transcription (lane 1). This indicated that all purified factors are functional for transcription in my highly purified transcription system, based on the previous study showing the requirement of all the factors for the activated transcription (Yu et al., 2001). Consistent with the results from the nuclear extract based assay, all the TRAP/Mediator exhibited comparable ability to support ER dependent transcription. (Fig. 5-10)

Fig. 5-9. Transcription activation by a model activator in a cell-free system reconstituted with purified factors. A. SDS-PAGE analysis of purified factors. Coomassie blue R250 staining of purified general initiation factors TFIIB, TBP, TFIIE, and TFIIF (lanes 1-4) and general coactivator PC4 (lane 5) were performed. Silver staining of the immunopurified FLAG-tagged multisubunit general initiation factors TFIID and TFIIH and RNA polymerase II (lanes 7-9) performed. Lane 6 shows molecular weight markers(SM). B. Activator-dependent transcription. Transcription was conducted with the purified components shown in panel A, GAL4-p53 and the DNA template described previously. Fold activation above the basal level is indicated at the bottom.

Transcription activation by a model activator in a cell-free system reconstituted with purified factors



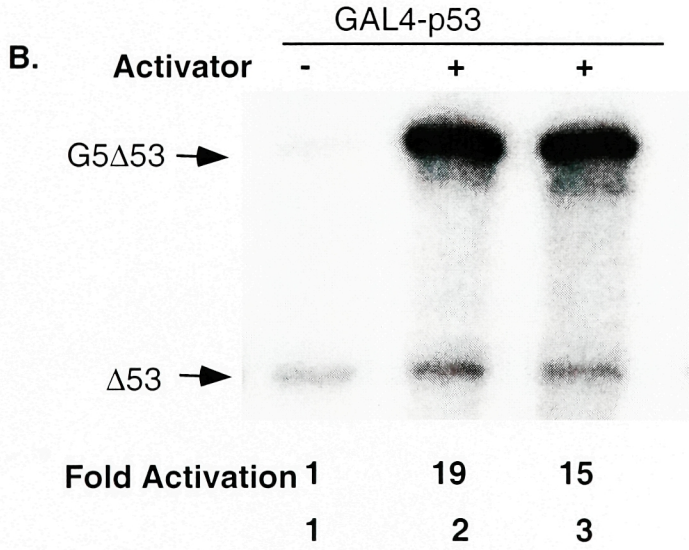
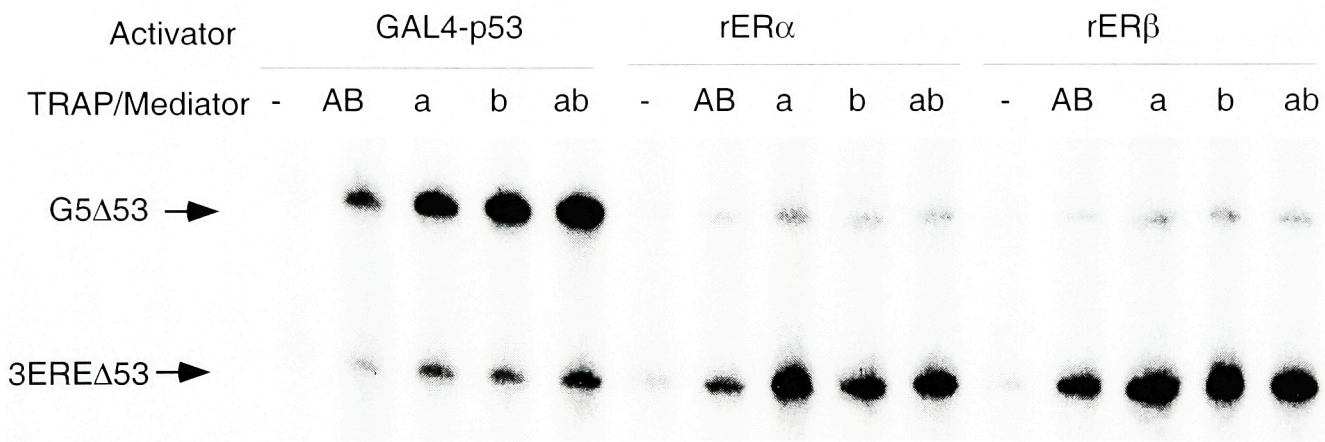


Fig. 5-10. Role for TRAP220 NR box in transcriptional activity of GAL4-ERβ LBDs in nuclear extract. In vitro transcription reactions contained purified general transcription factors, 4EREΔ53 G-less reporter template and G5Δ53 G-less control template as described in Materials and Methods. Additionally, the transcription reactions contained 10 ng of GAL4-p53 in lanes 1-5, 20 nM of recombinant ERα in lanes 6-10 and 20 nM of recombinant ERβ in lanes 11-15. Each purified TRAP/ Mediator complex (f:TRAP220AB in lanes 2,7, and 12; f:TRAP220a in lanes 3,8, and 13; f:TRAP220b in lanes 4,9, and 14;f:TRAP220ab in lanes 5,10 and 15) were added to transcription reactions. Relative transcription levels, determined by phosphorimaging, are indicated.

Effect of point mutation of TRAP220 on ER-dependent transcription in vitro transcription



Chapter 6

Identification of EDD/hHYD as an ER-interacting protein

6.1. Introduction

The hyd gene has originally been isolated from Drosophila mutants showing hyperplastic discs phenotype (Mansfield et al., 1994). The 280kDa drosophila HYD protein shares high homology with 100kD rat protein. This protein was found mainly in nuclear fraction (partly in cytosolic fraction) and expressed in all stages of development and all tissues (although varied levels of expression). Interestingly, this protein exhibited decreased expression in the mutants suggesting that it is a possible tumor suppressor.

Later, its human homologue was identified as EDD (E3 identified by differential display) by differential display for progestin-induced gene (Callaghan et al., 1998). In contrast to the previous implication of its potential function as a tumor suppressor, breast cancers showed higher expression of this gene. However, the overexpressed proteins in cancer cells could be the dominant negative mutants of EDD as reported in the case of p53. EDD contains HECT (homologous to E6-AP C-terminus) domain and can bind to Ubiquitin suggesting its function as E3 ligase in ubiquitylation. Subsequent study showed its E3 ligase activity both in vivo and in vitro

(Honda et al., 2002). Relevant to ubiquitylation, previous studies have shown the following: 1) ER is a ubiquitinated protein and level of ubiquitylation was different depending on ligands (Wijayaratne and McDonnell, 2001), 2) ER is regulated by proteasome pathway which may somehow be involved in ER transcriptional activity through modulation of cofactors (Lonard et al., 2000), and 3) E6-AP is an E3 ligase and showed coactivator activity for NR including ER (Nawaz et al., 1999). These studies, together with the finding that EDD/hHYD interacts with ERs, hinted at the possibility that this protein might play a role as an ER cofactor through its ubiquitin ligase activity. Here I describe the very preliminary studies on this possibility. EDD/hHYD interacts with both ERs in different manners. Overexpression of EDD/hHYD did show very modest effect on ER-driven transcription in transient transfection assays.

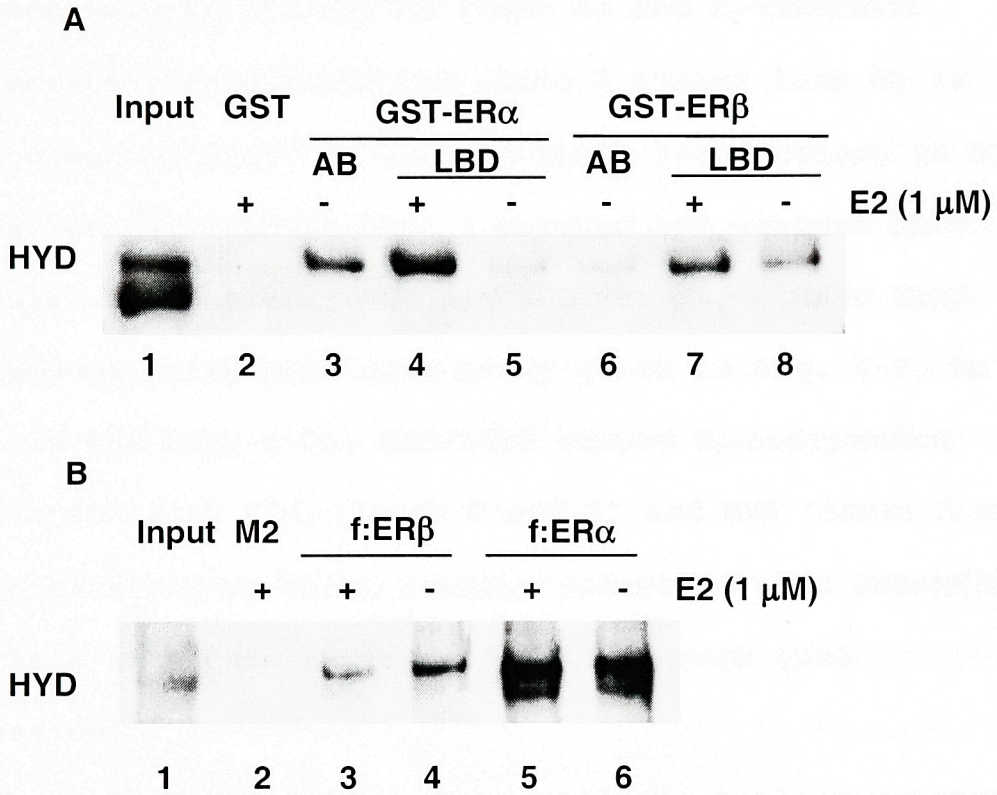
6.2. Results

6.2.1. HYD is an ER-interacting nuclear protein

To extend mass spectral analysis described in chapter 3.2, I employed western blot analysis with antibody

Fig. 6-1. EDD/hHYD is an ER-interacting nuclear protein. A. E_2-dependent interactions of EDD/hHYD with ERα and ERβ LBDs in nuclear extract. Immobilized GST (lane 2), GST-ERαAB (lane 3), GST-ERαLBD (lanes 4 and 5), GST-ERβAB (lane 6) and GST-ERβLBD (lanes 7 and 8) proteins were incubated with HeLa nuclear extract in the absence (-) or presence (+) of 1 μM E_2 and bound proteins were eluted and analyzed by SDS-PAGE and western blot with antibody against EDD/hHYD as described in Materials and Methods. One-tenth equivalent of the input nuclear extract is shown in lane 1. B. E_2-independent interactions between EDD/hHYD and ERs in HeLa nuclear extract. M2 agarose-immobilized FLAG-ERα (lanes 5 and 6) and FLAG-ERβ (lanes 3 and 4) were incubated with HeLa nuclear extracts in the absence (-) or presence (+) of 1 μM E_2 and bound proteins were eluted with FLAG peptide and analyzed by SDS-PAGE and western blot with antibody against EDD/hHYD as described in Materials and Methods. As a control, HeLa nuclear extract proteins bound to M2 agarose alone were analyzed in lane 2. One-tenth of input nuclear extract was analyzed in lane 1.

EDD/hHYD is an ER interacting nuclear protein

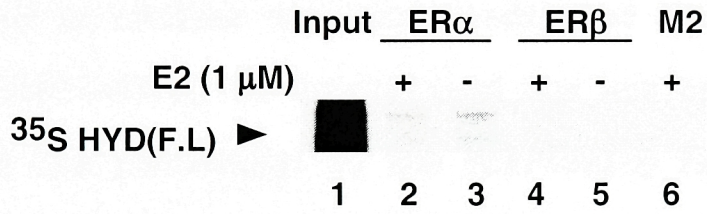


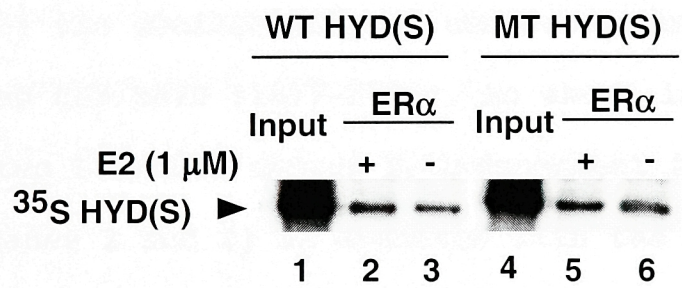
against EDD for the GST-pull down assays in Fig.4-1. As shown in Fig. 6-1A, EDD/hHYD exhibited interaction with GST-ERα AB (lane 3) and E_2-dependent interaction with GST-ERα LBD (lane 4 versus lane 5) in the nuclear extract. In case of ERβ, EDD/hHYD showed no detectable interaction with GST-ERβ AB (lane 6) and E_2-enhanced interaction with GST-ERβ LBD (lane 7 versus lane 8) in the nuclear extract. To examine these interactions in the context of full-length ERs, I carried out western blot analysis with antibody against EDD for the eluate from the immunoaffnity pull down assay shown in Fig. 4-7. As shown in the Fig. 6-1B. EDD/hHYD showed E_2-independent interaction with ERα (lanes 5 and 6) and ERβ (lanes 3 and 4). Interestingly, these results indicated that EDD/hHYD interacts with each ER in different manners (see discussion).

The studies described above utilized nuclear extracts as a source of EDD/hHYD, did not establish whether other factors were essential for these interactions. To further investigate this question, I employed the immunoaffinity pull down assays as described in chapter 4.2. The binding of ^{35}S-labelled full length EDD/hHYD to purified and immobilized full length ERα and ERβ was analyzed. As shown in Fig. 6-2A, full length EDD/hHYD showed the E_2-

Fig. 6-2. E_2-independent direct interactions between isolated EDD/hHYD and full-length ERs. A. Direct interactions isolated full length EDD/hHYD and full-length ERs. M2 agarose immobilized full length ERβ and ERα (lanes 2, 3, 4 and 5) and M2 agarose alone (lane 6) were incubated with ^{35}S-labeled (in vitro translated) full length EDD/hHYD in the presence (+; lanes 2 and 4) or absence (-; lanes 3 and 5) of 1 μM E_2. After washing, bound proteins were eluted with FLAG peptide and analyzed by autoradiography. Lane 1 shows one-twentieth of the ^{35}S-labeled full length EDD/hHYD input. B. Direct interactions truncated EDD/hHYD and full-length ERα. M2 agarose immobilized full length ERa (lanes 2, 3, 5 and 6) were incubated with ^{35}S-labeled (in vitro translated) truncated EDD/hHYD wild type (WT HYD (S)) and mutant (MT HYD (S))in the presence (+; lanes 2 and 5) or absence (-; lanes 3 and 6) of 1 μM E_2. After washing, bound proteins were eluted with FLAG peptide and analyzed by autoradiography. Lanes 1 and 4 show one-twentieth of each ^{35}S-labeled EDD/hHYD input.

Interaction between ER and EDD/hHYD





independent interaction with ERα, as observed with the nuclear extract. This indicated the direct interaction of EDD/hHYD with ERα. The interaction of the full length EDD/hHYD with ERβ, however, was not detectable. This might be due to the fact that the expression level of full-length EDD/hHYD protein was so low and the interaction of EDD/hHYD with ERβ was weaker than with ERα as observed in nuclear extract. Binding of truncated EDD/hHYD (1877-2799) to immobilized ERα was analyzed because of the following: 1) the difficulty in the expression of full length EDD/hHYD; 2) the truncated EDD/hHYD (1877-2799) contains a LXXLL motif among five LXXLL motifs (at amino acids 248, 1102, 1255, 1398, and 2428); and 3) the availability of the construct encoding the truncated EDD/hHYD (1877-2799). As shown in Fig. 6-2B this truncated EDD/hHYD showed E_2-independent interaction with ERα (lanes 2 and 3) as observed with the full-length EDD/hHYD. This result confirms that the isolated EDD/hHYD can directly interact with ERα.

To examine the role of the ubiquitin binding activity in the interaction of EDD/hHYD, binding of a mutant EDD/hHYD (1877-2799, C2768A) to immobilized ERα was analyzed. This mutant EDD/hHYD also showed E_2-independent interaction with ERα (lanes 5 and 6) as

observed with the corresponding wild-type fragment. This indicated that the ubiquitin binding activity of EDD/hHYD is not required for the direct interaction between the isolated fragment of EDD/hHYD and ERα.

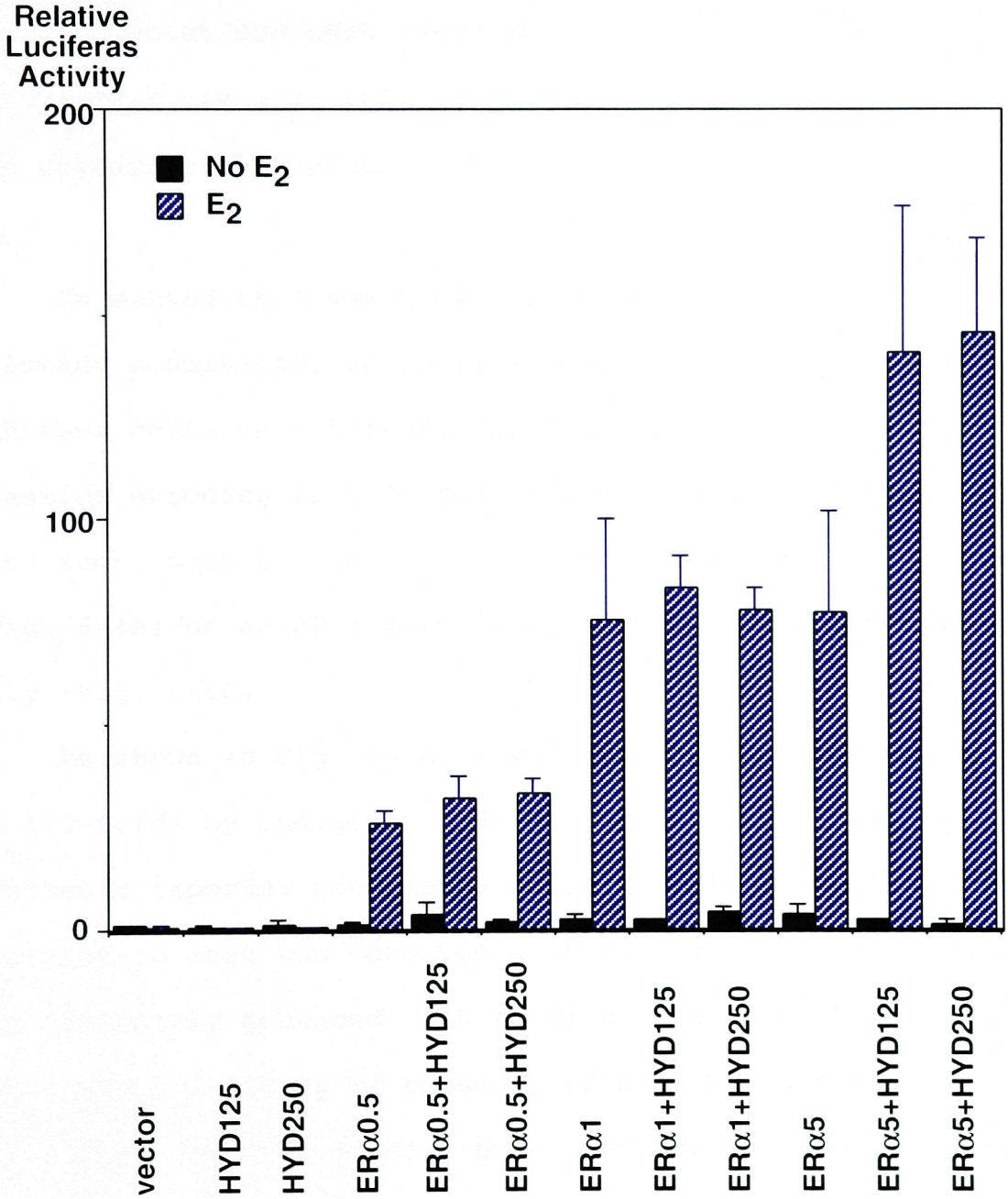
6.1.2. Effect of HYD on ER-mediated transcription

To examine role of EDD/hHYD in ER-mediated transcripton, I employed transient transfection assays. CV1 cell line is a simian kidney cell line which does not express ER and used commonly for the transient transfection assays with ER. CV1 cells were transfected with the indicated amounts of the plamids harboring ERα and EDD/hHYD, in combination with a reporter plasmid containing three ERE and core promoter, in the presence (E_2) or the absence of E_2 (No E_2).

In this assay (Fig. 6-3), a strong E_2 response (up to 30-fold induction at the 1 ng of ERα) was observed and this was further enhanced upon simultaneous transfection of the plasmids encoding full length EDD/hHYD. Due to the repressive effect by EDD/hHYD (1.4-fold at the concentration of 125 ng and 2.7-fold at the concentration of 250 ng of plasmids for EDD/hHYD) the net induction of E_2 response was enhanced up to 6-fold by EDD/hHYD. This result was very reproducible, however, it was not

Fig. 6-3. Role of EDD/hHYD in ERα-dependent transcription in CV1 cells. CV1 cells were transfected with indicated amounts of expression vectors for ERα and EDD/hHYD in combinations with 3ERE-luciferase reporter and control luciferase pRL-CMV as described in Materials and Methods. Cells were cultured in the absence (-) or presence (+) of the ligand, E_2 (10^{-8} M), and dual luciferase activities were measured 36 hr after transfection. Values (means ± SD of a representative experiment performed in duplicate) are plotted as a fold.

Role of EDD/hHYD in ERα-mediated transcription in CV1 cells



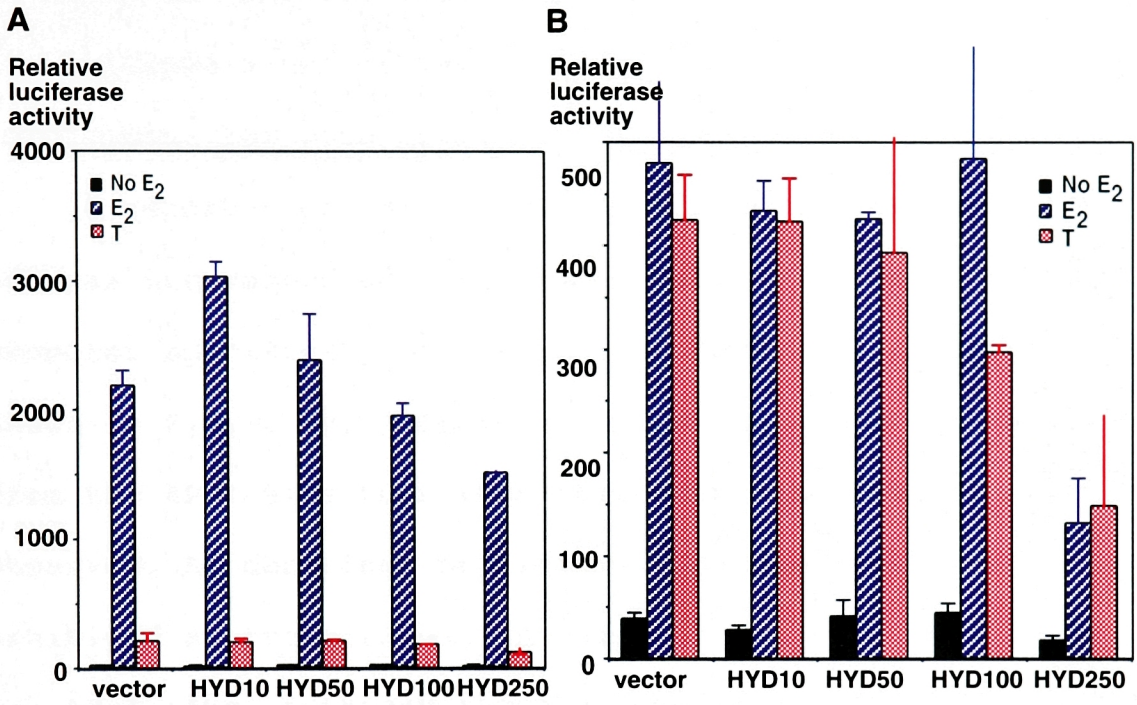
possible to confirm the expression of exogenous EDD/hHYD probably due to the limited sensitivity of the antibody against EDD/hHYD. Considering the observation of E_2-independent interaction of EDD/hHYD with ERα, this indicated that EDD/hHYD could be a potential cofactor for ERα through its dual role by acting as a corepressor in the absence of E_2 and as a coactivator in the presence of E_2.

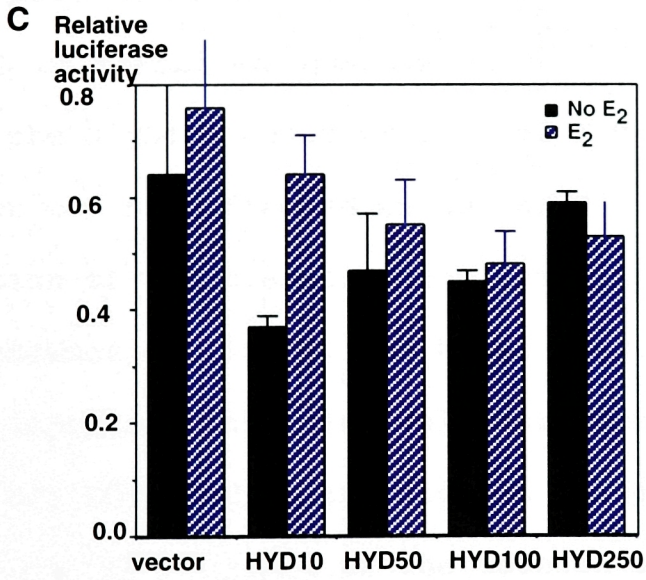
To extend this analysis with more physiologically relevant conditions, an Ishikawa cell line was employed. Ishikawa cells were transfected with indicated amounts of plasmids encoding full length EDD/hHYD in combinations with each reporter plasmid containing three ERE sites (Fig. 6-4A) or an AP-1 site (Fig. 6-4B) or core promoter only (Fig. 6-4C).

As shown in Fig. 6-4A, a very strong E_2 response (up to 100-fold) by endogenous ERs was observed with a synthetic reporter containing three ERE sites. In contrast to what was observed with CV1 cells, E_2 response was moderately enhanced (1.5 fold) at the lowest concentration (10 ng of plasmid) of EDD/hHYD and then attenuated (up to 1.5 fold) upon increasing concentration of EDD/hHYD essentially in a dose dependent manner although the level of ER-mediated transcription was not

Fig. 6-4. Role of EDD/hHYD in ER dependent transcription in Ishikawa cells. Ishikawa cells were transfected with indicated amounts of expression vectors for EDD/hHYD in combinations with luciferase reporter containing 3ERE (A), AP-1 (B) and no activator binding site (C) and control luciferase pRL-CMV as described in Materials and Methods. Cells were cultured in the absence (-) or presence (+) of the ligand, E_2 (10^{-8} M), and dual luciferase activities were measured 36 hr after transfection. Values (means ± SD of a representative experiment performed in duplicate) are plotted as a fold.

Role of EDD/hHYD in ER-mediated transcription in Ishikawa cells





affected by EDD/hHYD in the absence of ligand or in the presence of tamoxifen. This modest effect by exogenous EDD/hHYD might be due to the effect by endogenous EDD/hHYD in Ishikawa cells and the artificial effect by the multiple sites of ERE which might bypass the requirement for cofactors.

A reporter containing one AP-1 site was employed to address the second possibility as well as to examine promoter specificity of the role of EDD/hHYD, if any. As shown in Fig. 6-2B, a weaker E_2 response (up to 7-fold) from the AP-1 site than from three ERE sites was observed. As described previously, Ishikawa cells exhibited a strong tamoxifen response (up to 7-fold) from the AP-1 site. Although the absolute level of transcription activity from AP-1 site in the presence of either E_2 or tamoxifen appeared to be attenuated by 4-fold at the highest concentration of EDD/hHYD the net induction was not affected by EDD/hHYD due to the attenuation of the transcription activity from AP-1 site in the absence of ligand. The basal activity from a control reporter containing only core promoter (Fig. 6-4C) was not affected at the highest concentration of EDD/hHYD in the presence or the absence of E_2. This indicated that the reduction in the level of activity

from the AP-1 site could be mainly due to the reduction in the activated-transcription not the basal (activator-independent) activity.

Chapter 7

DISCUSSION

ER mediates estrogen signaling through regulation of gene expression via direct and/or indirect ways by a variety of mechanisms as described above. Considering the broad functions of estrogen, the existence of only two ERs may require such diversity and complexity of ER action mechanisms. Cofactors are considered as ultimate integrators and processors of these various mechanisms. The diversity of cofactors provides (1) the interfaces to accommodate various regulatory information imparted by enhanceosomes, chromatin structure, and general transcription machinery; (2) the means to integrate secondary signaling pathways with estrogen signaling via cross-talk; and (3) mechanisms, through cofactor modulation, to dictate the specificity of ER function depending on the different cell types, promoters and ligands. Therefore, understanding the cofactors that regulate ER function is particularly interesting, and important.

With respect to this notion, I initially proposed the existence of more novel cofactors involved in ER function, on the basis of the existence of receptor subtypes (α and β) with different tissue distributions, functions and ligand responses; cell and promoter context-dependent functions of resident (AF-1 and AF-2)

activation domains; and the existence of selective estrogen receptor modulators (reviewed in McDonnell, 1999).

The object of this thesis work was to identify the ER-interacting proteins (presumptive cofactors) that are involved in specificity of subtypes of ER and/or cell types. Here I described the purification and partial characterization of ER LBD-interacting proteins in an E_2-dependent manner.

7.1. Cell type specificity of ER-interacting proteins

The apparent lack of major differences in the electrophorectic migration patterns of the polypeptides interacting with GST-ERα LBD and GST-ERβ full-length in the cell types that I used was quite surprising. However, cell type specific functions of ERs might be mediated through proteins that weakly interact with ER LBDs and/or a repertoire of cell type-specific proteins that interact with the AF-1 domains of ERs although I have not pursued in sufficient depth to ascertain. Therefore, the initial hypothesis could be still valid. In support of this idea, the recent study showed the difference in the expression

level of SRC-1 between MCF-7 and Ishikawa cells contributes the difference in cell type-specific ER function in these cell lines (Shang et al., 2000).

7.2. Subtype Specificity of ER functions

Although previous studies have reported activation by ERα in cell free systems (Kraus and Kadonaga, 1998; Wu et al., 1999), this is the first report to compare the intrinsic activities of ERα and ERβ in a fully-defined system reconstituted with essentially homogeneous factors. Our observation that ERβ is more potent than ERα in the TRAP/Mediator-dependent assay system is somewhat surprising in view of reports that ERα is generally more active than ERβ in transfection assays with ectopic reporters (reviewed in Hall and McDonnell, 1999; Nilsson et al., 2001; Pettersson and Gustafsson, 2001).

However, the in vitro transcription data correlate directly with my observation that intact ERβ has a higher affinity for TRAP/Mediator than does intact ERα. Furthermore, promoter and cell context-dependent effects of ER AF-1 and AF-2 domain are well established (reviewed in Hall and McDonnell, 1999; Nilsson et al., 2001;

Pettersson and Gustafsson, 2001). Especially relevant to the present findings, an ERβ lacking the AB domain is much more active than an ERα lacking the AB domain in certain (e.g. HeLa) cell types (Hall and McDonnell, 1999). This reflects the well established synergy of ERα AF-1 and AF-2 domains (Hall and McDonnell, 1999; Nilsson et al., 2001; Pettersson and Gustafsson, 2001) and an apparent inhibitory effect of ERβ AF-1 on ERβ AF-2 function (Hall and McDonnell, 1999). Since ERα AF-1 function has been shown to involve site-specific phosphorylation and recruitment of cofactors not present in our in vitro assay (Watanabe et al., 2001 and references therein) my results likely reflect an intrinsic AF-2 function mediated through TRAP/Mediator interactions.

In contrast to this thesis study, but consistent with the cell-based studies, a recent report showed that ERα is more potent than ERβ in an in vitro transcription assay with chromatin template but not with a naked DNA template (Cheung et al., 2003). Cheung et al also demonstrated that A/B region of ERα but not ERβ contained potent transcriptional activity with chromatin template based assays not with naked DNA based assays (Cheung et al., 2003). Thus, it was suggested that the stronger

activity of ERα than that of ERβ shown in their study could be mainly due to the stronger AF-1 activity of ERα than that of ERβ (Cheung et al., 2003). Considering the fact that Cheung et al employed the extract-based systems for both chromatin assembly and transcription assays, the difference in the transcriptional activity of ERα and ERβ observed in their study could be significantly mediated via additional factors, which present in the extract that support ERα AF-1 activity at the step of antirepression of the chromatin, rather than the intrinsic transcriptional attributes (ERβ>ERα) mediated via GTFs (TFIIB, TFIID, TFIIE, TFIIH, polII, and PC4) and TRAP/Mediator as seen in this thesis study. Also of importance is my observation that the differential affinities of TRAP/Mediator for intact ERs (β>α) are reversed when the AB domains are absent (α>β). This clearly indicates an ability of the AB domains to modulate interactions of the LBD (AF-2) domains with TRAP/Mediator (and probably other cofactors), although it is not clear whether this reflects an inhibitory effect of the ERα AB domain on ERα AF-2 interactions and/or a stimulatory effect of the ERβ AB domain on ERβ AF-2 interactions with TRAP/Mediator. A very recent report on an inhibitory activity of part of the ERα AB domain

strongly supports the first possibility (Metivier et al., 2002). Additionally, the results reported here likely reflect intrinsic (intramolecular) effects of the AB domain(s), rather than effects of additional AB domain-interacting factors, since similar effects were observed with unpurified (nuclear extract) and highly purified TRAP/Mediator. Although both Freedman and colleagues (Burakov et al., 2000) as well as I failed to see differential interactions of ERα and ERβ with isolated TRAP220, Treuter and colleagues (Warnmark et al., 2001) have reported stronger interactions of isolated TRAP220 (and TRAP220 fragments) with ERβ than with ERα. Although the basis for these discrepancies is not clear, the latter results are consistent with our observation of stronger ERβ interactions with TRAP220 in the more physiological context of the functional TRAP/Mediator complex. Given that other AF-2-interacting coactivators such as p160/SRC members show little subtype selectivity in ER binding (Burakov et al., 2000; Warnmark et al., 2001) and as discussed by Warnmark et al. (Warnmark et al., 2001), the differential binding of TRAP/Mediator to ERα and ERβ has significant implications for differential functions of these receptors in diverse tissues that could show modulations of TRAP/Mediator components. The

differential recruitment of these factors may also contribute to the tissue-selective action of selective ER modulators. Finally, apart from a number of other interacting factors (reviewed in Nilsson et al., 2001) implicated in ER functions, it will be important to further characterize the additional ER-interacting polypeptides identified here (Fig. 3-2) for possible subtype-specific functions and interactions with TRAP/Mediator, other cofactors, and the general transcription machinery.

7.3. TRAP/Mediator is a Bona Fide Estrogen Receptor Coactivator

Following earlier reports of ligand-dependent interactions of ERs with the isolated TRAP220 subunit of the multicomponent (circa 25 subunit) TRAP/Mediator complex (Burakov et al., 2000; Warnmark et al., 2001; Yuan et al., 1998; Zhu et al., 1999), this thesis study provides concrete evidence for direct TRAP220- and ligand-dependent interactions of ERs with the intact TRAP/Mediator complex and a direct role for the complex

in ER function through the general transcription machinery.

While a role for the complete TRAP/Mediator in ER function had been inferred from TRAP220 binding studies (Burakov et al., 2000; Warnmark et al., 2001; Yuan et al., 1998; Zhu et al., 1999), and from the precedents established with TR and VDR (Fondell et al., 1996; Rachez et al., 1998), the concurrent failures (see above) to show any interactions of the intact TRAP/Mediator complex with ERs had led others to speculate that class I (steroid hormone) nuclear receptors might utilize different cofactors from TRAP/Mediator (Burakov et al., 2000). This thesis work provides the first evidence for the involvement of the intact TRAP/Mediator complex in the function of class I (steroid hormone) nuclear receptors.

Consistent with the earlier results, and with structural conservation of the AF-2 domain implicated in ligand-dependent interactions of many cofactors, this thesis work shows ligand-dependent interactions of TRAP/Mediator both in nuclear extracts and in purified form with both ERα and ERβ LBDs. The latter results establish that these interactions are direct, while analyses with extracts from TRAP220$^{-/-}$ fibroblasts

establish an essential role for TRAP220 in these interactions. Consistent with their role in TR and VDR interactions, the TRAP220 LXXLL motifs (most notably NR box 1) have been implicated in ligand-dependent ERα and ERβ interactions with isolated TRAP220 (Burakov et al., 2000; Warnmark et al., 2001). Thus, although reported ligand-independent interactions of ERs with other isolated TRAPs (Burakov et al., 2000) could be relevant, they are insufficient for demonstrable interactions of the entire TRAP complex with ERs. Related, I did not observe any direct interactions of TRAP/Mediator with ER N-terminal AB domains.

Importantly, the physiological relevance of the in vitro interactions is substantiated by our demonstration of an in vivo association of the entire TRAP/Mediator complex with an ERα derivative lacking the AB domain. Along with the in vitro data, this observation makes improbable the suggestion (Burakov et al., 2000) that TRAP220 may mediate ER function independently of many or all of the other TRAP/Mediator subunits. In a further analysis of the significance of TRAP/Mediator interactions, and perhaps most importantly, this thesis work demonstrates that the purified TRAP/Mediator complex markedly enhances ER function on DNA templates both in

nuclear extracts and in a system reconstituted with highly purified factors. This firmly establishes a direct function for TRAP/Mediator in mediating the action of ERs on target promoters in conjunction with the general transcription machinery.

A recent report utilizing in vitro transcription assays with a chromatin template showed only a modest effect (1.3 to 2 fold on activated transcription by ERα) on ER-dependent transcription upon TRAP/Mediator depletion (Acevedo and Kraus, 2003). This result is contrast to my results, which showed the significant effect (4 to 7 fold) by TRAP/Mediator on the activated transcription by ERs or GAL4-ER LBDs. The difference between my study and the study by Acevedo and Kraus might be due to different depletion procedures such as using different antibodies. Another possibility for the different results between my study and their study could be difference in templates (naked DNA in my study versus chromatin template in their study) although this is quite unlikely considering an earlier study showing the significant effect on HNF-4 dependent transcription upon TRAP/Mediator depletion in nuclear extract-based in vitro transcription assays with both DNA and chromatin templates (Malik et al., 2002). Most of all, the

interpretation of the study by Acevedo and Kraus is difficult due to the the lack of restoration experiment by adding exogenous TRAP/Mediator complex and possible complement by Drosophila Mediator existing in S-190 extracts.

7.4. TRAP220 is an essential anchor for ER-TRAP/Mediator interaction

Although a number of previous studies suggested the potential involvement of the TRAP220 subunit in ER function (Burakov et al., 2000; Warnmark et al., 2001; Shang et al., 2000; Zhu et al., 1999; Yuan et al., 1998), these studies employed only isolated TRAP220 or fragment of TRAP220. Thus, these studies were not able to score the function of TRAP220 in the context of intact TRAP/Mediator. To address this issue, TRAP220-/- MEFs were utilized to examine the roles for TRAP220 in the interaction and function of TRAP/Mediator in ER-driven transcription. This study was based on the previous observation that the residual Mediator subunits can form a functional complex for certain activators such as GAL4-VP16 (unpublished observation by Chao-Xing Yuan). As

mentioned above, the loss of TRAP220 abolished the ER-TRAP/Mediator interaction in nuclear extract indicating a role for TRAP220 as an essential anchor for the strong TRAP/Mediator-ER interaction. However, and surprisingly, transient transfection assays with TRAP220-/- MEFs revealed that ER-driven transactivation was reduced only modestly (2 fold) by deletion of TRAP220, in contrast to what was observed with TR. This was unexpected considering the significant effect on ER-TRAP/Mediator interaction by deletion of TRAP220. This discrepancy might be due to the existence of redundant mechanisms that likely compensate for potential defects in ER function by the loss of TRAP220 in MEFs. This could be 1) the presence of redundant cofactors that play similar roles as TRAP220 in MEFs and/or 2) the absence of natural constraints (such as a natural chromatin structure) that particularly impose a requirement for TRAP220 to overcome and/or 3) physiologically irrelevant cellular environment for ER function given that MEFs do not express ERs and/or 4) non-physiological template containing multiple ERE sites.

A consideration of studies with PPARγ, another receptor that shows ligand-dependent interaction with TRAP220, exemplified this point. Thus, transient

transfection assays with MEFs failed to show a significant defect in PPARγ-driven transcription in the absence of TRAP220 (unpublished observation by Chao-Xing Yuan), however, adipogenesis differentiation assay revealed the absolute requirement of TRAP220 for PPARγ-induced adipogenesis differentiation and for endogenous PPARγ target gene expression (Ge et al., 2002). Moreover, the GST pull-down assays with TRAP220-/- nuclear extracts showed that TRAP220 is an essential for strong interactions of PPARγ with the TRAP/Mediator, as observed for ER. These results strongly support the above possibilities and necessitate more physiological cell-based assays that employ cells in where ER normally function in the expression of natural target genes.

Based on previous studies in assaying the function of TRAP/Mediator in ER dependent transcription, I employed cell-free assays, as an alternative approaches, to assess the role of TRAP220 in ER-dependent transcription, particularly in the context of intact TRAP/Mediator. However, TRAP/Mediator complex lacking TRAP220 showed no defect in ER-dependent transcription in this particular assay. This unexpected observation, together with the observation of the requirement of TRAP220 for the ER-TRAP/Mediator interaction, led to the

speculation that 1) whereas TRAP220 play a pivotal role in mediating ER-TRAP/Mediator interaction, this may not be essential for TRAP/Mediator to support ER dependent transcription at the naked DNA template in vitro and 2) factors other than ER may be able to recruit TRAP/Mediator lacking TRAP220 to the promoter through other subunits under the ambient conditions in cell-free transcription assays. The dispensability of TRAP220 for TRAP/Mediator function in activated transcription in cell-free transcription assays has been suggested by previous studies in cell free systems showing 1) that TRAP/Mediator lacking TRAP220 can support activated transcription by certain activators such as GAL4-VP16 and HNF-4 (unpublished observation by Sohail Malik) and 2) that a preparation of PC2 containing no detectable levels of TRAP240, 230, 220, can support activated transcription by GAL4-VP16, GAL4-AH and HNF-4 as well as intact TRAP/Mediator (Malik et al., 2000). Moreover, and importantly, The possibile existence of other factors (not activator) that can recruit the residual (TRAP220-deficient) TRAP/Mediator to the promoter through other subunits of TRAP/Mediator was indirectly suggested by the previous studies showing an obligate role of the TRAP/Mediator in supporting basal (activator-independent)

transcription in depleted extracts (Baek et al., 2002; Mittler et al., 2001). More direct evidence for this was provided by a subsequent study showing that TRAP/Mediator was recruited to the promoter in the absence of the activator HNF-4 (Malik et al., 2002).

In addition to the basal effect, TRAP/Mediator also supports ER-activated transcription. The question then arises as to how TRAP/Mediator supports ER-activated transcription. One possible answer to this question is that although ER might not be able to directly recruit TRAP/Mediator due to lack of TRAP220, residual TRAP/Mediator lacking TRAP220 could function in ER-activated transcription via indirect recruitment through other factors.

Thus, the failure to show a role of TRAP220 in the function of TRAP/Mediator to support ER-dependent transcription using cell-free assays might be due to an intrinsic limitation of cell-free assays. Therefore, it will be important to develop assays that impose more physiological conditions before taking the conclusions regaring the roles for TRAP220 in the function of TRAP/Mediator to support ER-dependent transcription.

7.5. Role for TRAP220 NR boxes

The LXXLL motif was first identified as a signature sequence motif, in other nuclear receptor coactivators, that is necessary and sufficient for direct interactions with NRs (Heery et al., 1997). This motif has been found in most NR cofactors (Heery et al., 1997). Furthermore, this motif functions as a specificity determinant for interactions between NRs and cofactors (McInerney et al., 1998). For example, consistent with this notion, it was indicated by others that the second (C-terminal) LXXLL motif (NR box2) of TRAP220/DRIP205 showed the interaction with TR (Yuan et al., 1998) and VDR (Rachez et al., 1998), whereas the the first motif (more N-terminal) LXXLL motif (NR box1) showed a preferred interaction with ERs (Burakov et al., 2000; Warnmark et al., 2001). However, in contrast to these previous reports, this thesis study revealed that ER LBDs prefer TRAP220 NR box2 to NR box1 in the isolated TRAP220 AB. The difference may be due to the different assay condition. Interestingly, full-length ERβ did not show any preference in the context of intact TRAP/Mediator and both NR boxes were found to be necessary and sufficient for strong ER-TRAP/Mediator interaction. This, together with previous

reports, indicated that each NR box is sufficient for ER-TRAP220 interaction but not sufficient for efficient ER-TRAP/Mediator interaction. This suggested cooperative action of two NR boxes in ER-TRAP/Mediator interaction.

In consistent with these results, transient transfection assays with MEFs revealed that exogenous expression of full-length TRAP220 harboring mutations in either or both NR boxes failed to restore the defect in ER-driven transcription. The cell-free assays both with crude nuclear extract and with purified factors showed that TRAP/Mediator complex containing TRAP220AB NR mutants exhibited only very modest defects (1.5 to 2 fold) in ER-dependent transcription. This is quite surprising, considering previous observation of more significant effects of the same TRAP220AB NR mutants on the activated-transcription by other nuclear receptors such as TR (unpublished observation by Sohail Malik and Mohamed Guermah) or PPARγ (unpublished observation by Mohamed Guermah) in cell-free transcription assays.

These modest effects of TRAP/Mediator containing TRAP220AB NR mutants could be due to the same reasons for the results of many analyses with TRAP/Mediator lacking TRAP220, as addressed in the previous section (chapter 6-4), provided that mutations of either or both NR boxes of

TRAP220 are functionally equivalent to complete loss of TRAP220. However, and more importantly, this functional outcome could be due to the residual interactions between ER and TRAP/Mediator containing TRAP220AB harboring mutations in either or both NR boxes. These weak but significant interactions of TRAP/Mediator containing TRAP220AB NR mutants with ERs could be enough for function. In support of this idea, the TRAP220 NR boxes are dispensible for the physiological function of PPARγ which absolutely requires TRAP220 (unpublished observation of Kai Ge).

7.6. Identification of EDD/hHYD as an ER-interacting protein

The previous studies on the EDD/hHYD summarized in the chapter 6, pointed out strong relevane of EDD/hHYD in ER function. This thesis work provided evident to support this possibility although the data presented here is yet preliminary.

EDD/hHYD interacts with each ER in subtype-dependent manners. EDD/hHYD interacts with GST-ERα AB but not with GST-ERβ AB. This suggested the possible role of EDD/hHYD

in the AF-1 activity of ERα. The isolated ERα LBD showed E_2-dependent interaction with EDD/hHYD, whereas the isolated ERβ LBD showed E_2-enhanced interaction. The reason for the less E_2-dependency of the interaction of EDD/hHYD with ERβ LBD than with ERα LBD has yet to be known. It could be due to potential involvement of other factors in nuclear extract in the interaction of EDD/hHYD with each ER LBD and/or intrinsic properties of ER LBDs such as structural difference of each ER LBD. Although it needs to be established the difference in E_2-dependency of the interaction between EDD/hHYD and each ER LBD suggested the potential involvement of EDD/hHYD in the subtype-specific response of ER to different ligands. In contrast to what was observed with TRAP/Mediator, EDD/hHYD interacts with full length ERα much stronger than with full length ERβ, as observed with ER LBDs although to somewhat lesser extent. The reason for the stronger interaction of EDD/hHYD with full length ERα compared to full length ERβ has yet to be known. However, it appeared to be same with the isolated EDD/hHYD this might reflect the intrinsic difference in ERα and ERβ rather than the other factors in the nuclear extract.

Interestingly, EDD/hHYD interacts with full length ERs in E_2-independent manners. The reason for the loss of

E_2-dependency has yet to be known, however, these resuts indicated the modulatory effect of AB regions of ERs, via the intrinsic properties of AB regions of ERs, on the interaction of EDD/hHYD with corresponding LBDs, as suggested from the studies with TRAP/Mediator.

To examine the potential role of EDD/hHYD as a cofactor for ERs, the transient transfection assays with two different cell types were carried out. In CV1 cells, the transfection of exogenous EDD/hHYD resulted in the apparent net induction of ERα-mediated transcription through its dual action by stimulating E_2-dependent activity of ER and repressing E_2-independent activity. In contrast to this, Ishikawa cells exhibited the reduction in the activity from the multiple ERE sites as well as single AP-1 site upon the transfection of exogenous EDD/hHYD in a ligand-independent manner. The discrepancy in the results with two cell lines could be due to the intrinsic properties of these cell lines, particularly the level of endogenous EDD/hHYD protein as well as ERs. For example, if Ishikawa cells overexpress endogenous EDD/hHYD one might not be able to see the effect of exogenous protein. A report that was published during this thesis work provided evidence that EDD functions as a coactivator for PR and VDR but not for ER (Henderson et

al., 2002). Henderson et al also reported that neither the ubiquitin binding domain nor the E3 ligase activity was required for such cofactor function (Henderson et al., 2002).

7.7. Identification of ER LBD-interacting proteins independent of TRAP/Mediator

As detailed in Fig. 3-3, circa 12 polypeptides were identified as ER LBD-interacting proteins (ERLIPs). Although the functional relevance of their interactions with ERα LBD remains to be established there is some evidence suggesting their potential involvement in ER functions.

1) DNA-dependent Protein Kinase (DNA-PK)

The largest protein among the ERLIPs was identified as the 450 kDa DNA-PK catalytic subunit. The possible role for this protein in modulation of ER function was first suggested by an early report showing its ability to phosphorylate ERα in vitro (Arnold et al., 1995). Additional evidence for the potential link between DNA-PK and NR function came from the studies showing an

interaction of DNA-PK with certain cofactors such as TRBP and GCN5 (Ko et al., 2000). TRBP interacts with CBP/p300 and DRIP130/hSUR2, a component of the TRAP/Mediator and activates transcription by TR and ER (Ko et al., 2000). This study also demonstrated DNA-independent phosphorylation of TRBP by DNA-PK, although its functional relevance was not shown (Ko et al., 2000). Furhtermore, phosphorylation of GCN5 by DNA-PK was found to down regulate the HAT activity of GCN5, resulting in an inhibitory effect on transcription.

Based on these results, one can propose two possible mechanisms by which DNA-PK modulate ER functions: 1) direct roles for DNA-PK in modulation of ER activity through its phosphorylation of ERα and 2) the indirect roles via interactions with other cofactors by facilitating recruitment and/or modulating cofactor activity through its kinase activity.

2) Transformation/transcription domain-associated protein (TRRAP)

The second largest protein in ERLIPs was identified as the TRRAP component of GCN5/PCAF complexes (Vassilev et al., 1998). This is consistent with the previously documented E_2-dependent recruitment of PCAF to ER-

regulated target genes (Shang et al., 2000). Additionally, there is a report suggesting the involvement of TFTC, one of GCN5/PCAF complexes, in ERα dependent transcription through direct interaction of TRRAP with ERα (Yanagisawa et al., 2002). This study also provided evidence that TRRAP is involved in E_2-dependent cell growth of breast cancer cells. However, the data from in vitro transcription assays were not so convincing because this study failed to demonstrate the activity by TFTC complex by itself and the effect on cell growth by antisense TRRAP could be a secondary effect.

3) RNA Polymerase II subunit

Although the direct interaction of RNA pol II with ER has not been indicated heretofore, RNA pol II has been shown to be associated with TRAP/Mediator in certain cases. Therefore, it is highly possible that RPB2 might indirectly interact with ER LBD via TRAP/Mediator.

4) Striatin, S/G_2 Nuclear autoantigen (SG2NA), Zinedin, Protein phosphatase 2A (PP2A) and hMOB1/Phoecin

These proteins were shown to form a PP2A holoenzyme complex with calcium-independent, okadaic acid-sensitive phosphatase activity (Baillat et al., 2001; Moreno et

al., 2001; Moreno et al., 2000). These studies suggested that Striatin, S/G_2 Nuclear autoantigen (SG2NA), Zinedin, and hMOB1/Phoecin form a novel B subunit of PP2A holoenzyme.

PP2A is a heterotrimeric serine/threonine phosphatase consisting of structural (A) subunit, catalytic(C) subunit and regulatory (B) subunit. Existence of several types of B subunits-B, B′, B″, B′″ could produce many different holoenzyme assemblies which can modify specific substrates in distinct cellular environment. This structural diversity and complexity of PP2A could create the multifunctional nature of PP2A that is involved in cellular metabolism, neuronal signaling, development, cell cycle regulation and viral transformation.

Striatin, SG2NA and Zinedin have been shown to be a striatin family due to their high homology. These proteins contain multiple protein-protein interacting domains including a caveolin-binding domain, potential coiled-coil structure, a calmodulin-binding domain, a membrane-binding domain and a WD-repeat domain (Castets et al., 2000). With respect to these structural features these proteins have been thought to function as scaffolding proteins by assembling a large number of proteins into a complex (Castets et al., 2000). Thus,

these proteins target other proteins to certain cellular compartments. Additionally, their binding ability to calmodulin in the presence of calcium suggesting their function in or depending on calcium signaling (Bartoli et al., 1998) (Castets et al., 2000). Another interesting feature of these proteins is that they expressed primarily in the central nervous system suggesting their function in the brain.

Among those, SG2NA was originally isolated as an autoantigen in a human cancer patient (Muro et al., 1995) and localized to the nucleus although it is primarily localized to the cytosol and the membrane. As its name indicated its expression peaks during the S and G2 suggesting its involvement in cell cycle (Muro et al., 1995).

Striatin was found in neurons of mammalian basal ganglia and cranial and spinal motor nuclei specifically, neuronal dendrites (Castets et al., 1996; Moqrich et al., 1998; Salin et al., 1998). Different from SG2NA, striatin was found in the cytosol and in membranes (Castets et al., 1996) and contains two polybasic domains that are absent in SG2NA and may facilitate association with the post-synaptic membrane (Castets et al., 1996). Its physiological function was further supported by the

observation that downregulation of striatin in vivo using antisense oligonucleotides decreased locomotor activity and reduced growth of dendrites in vitro (Bartoli et al., 1999). These results suggested that striatin with the PP2A A/C heterodimer target to a cellular microenvironment in which it may play a role in the modulation of calcium-dependent neuronal signaling and possibly remodeling of the cytoskeleton.

There are a couple of reports suggesting the involvement of these proteins in regulation of transcription. An early report provided evidence that PP2A catalytic subunit can potentiate transcription from the AP-1 sites (Alberts et al., 1993). SG2NA was suggested to contain transcriptional activity (Zhu et al., 2001). Although their involvement in estrogen signaling has not yet been clearly demonstrated there is a report showing that direct interaction of PP2A catalytic subunit with ERα and increase in ER-driven transcription upon treatment of okadaic acid via inducing ER-MAPK interaction (Lu et al., 2003). However, it is more likely that PP2A might involve in ER-driven transactivation rather than inhibition based on the observation of the ligand-dependent interaction of PP2A with ERα. Moreover, the interpretation of the data on ER-

driven transactivation with okadaic acid is difficult based on the following: 1) the effect of okadaic acid could be secondary effect and 2) one should include proper control for the specificity such as by showing that other activator dependent transcription is not affected.

5) Calmodulin-dependent kinase II (CaMKII)

CaMKII, similar to CaMKI, is widely expressed throughout the body whereas CaMKIV is relatively restricted to the brain, T-lymphocytes and post-meiotic male germ cells. Its dual roles in transcription regulation have been suggested by a report showing that CaMKII phosphorylates ATF-1 at Ser63 and activate its transcriptional activity by enhancing the interaction with CBP while it phosphorylates CREB at Ser142 and inhibit its transcriptional activity by reversing the activation of CREB by destabilizing the association between CREB and CBP (Shimomura et al., 1996). Similar to PP2A this kinase may be more relevant to the nongenomic actions of ER rather than genomic actions. However, this possibility remains to be investigated.

7.8. Perspectives

Estrogen involves diverse physiological processes in various tissues. Thus, natural estrogen withdrawl in women in the postmenopausal period has caused pathological symptoms called postmenopausal syndrome. Particularly, today the increasing life span has generated increasing population of women in the postmenopause stage and longer postmenopausal period of their lives meaning that more women would suffer from postmenopausal syndrome for longer period. Estrogen also has known to be involved in the carcinogenesis of breast and uterine. Therefore, estrogen has been getting considerable clinical and pharmaceutical attention due to its significance in women's health. In particular, it is of great interest to develop new SERMs as substituents for hormone replacement therapy that retain the beneficial effects of estrogen, but lack of higher risk of breast cancer or uterine cancer. Understanding the molecular mechanism of estrogen action and its receptor ER can eventually be foundation for the discovery of proper SERMs to treat or prevent estrogen-related diseases.

A prerequisite for understanding the molecular events underlying the ER-mediated estrogen action is the identification of all the players involved. In this respect I believe that my thesis work has contributed to scientific progress by identifying and partially characterizing some of the ER-interacting proteins (presumptive cofactors).

Future studies must elucidate the physiological roles for TRAP/Mediator in ER function. As reviewed in the previous sections, TRAP/Mediator would link the diverse gene-specific transcriptional factors and basal transcription machinery and provides accommodating surfaces to transcription apparatus with its modular and dynamic organization upon diverse regulatory signals. In this way, TRAP/Mediator could act ultimately (1) to assemble various regulatory signals from enhanceosomes into an integrated synergistic response to the basal transcription machinery at the promoter of a given gene and (2) to coordinate afferent signals from various signaling pathways in the context of the cellular environment into an incorporated differential response to the cell and tissue of a whole organism. In view of the first aspect, future studies must be directed towards illumination of the molecular mechanisms by which

TRAP/Mediator connects ERs and the components of basal transcription machinery. In view of the second aspect, it will be very important and interesting to study the involvement of TRAP/Mediator in various signaling pathways involved in differentiation and/or development. In other words, the physiological relevance of TRAP/Mediator in ER function, as observed in PPARγ-induced adipocyte differentiation should be investigated. For example, RNAi with breast cancer cells to see potential relevance of amplification of TRAP220 in breast cancer. Generation of tissu-specific conditional TRAP220 knock-out mice would provide more insights of the role of TRAP220 and TRAP/Mediator complex in the mammalian physiology including nuclear receptor function. Furthermore, such physiological approaches could assess the functional relevance of absolute requirement of TRAP220 in ER-TRAP/Mediator interaction.

Other future studies will have to characterize the function of ER-interacting proteins other than TRAP/Mediator in both nongenomic and genomic actions of ERs, elucidate the molecular mechanism leading to ER function through these proteins, and address questions of possible subtype specificities of these proteins.

Another question provoked by the existence of diverse cofactors for ER function is the functional relationship among these proteins whether they function exclusively, cooperatively, or redundantly.

Chapter 8

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