Proteomics of androgen receptor co-regulators in prostate cancer

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To my lovely grand father
and my supportive parents and sister
One of the life's great pleasures is being with warm and caring people

Abstract

The androgen receptor (AR) is a ligand-dependent transcription factor. Binding of androgens induces a conformational change and subsequently binding of the receptor to androgen receptor elements (ARE) on the genomic DNA. AR activity and specificity is additionally modulated by co-regulators that are recruited upon ligand binding. So far, the precise role of co-regulators in mediating AR function in different state in growth, aging, development and diseases is poorly understood. Furthermore, studies have shown that post-translational modification of the AR can affect AR activity. Androgen receptor is also one of the driving forces of prostate cancer. Therefore detail knowledge about the function of AR co-regulators is of urgent need.

Once we know which co-regulators are essential for cancer development, one option to treat the cancer cells will be inhibition of the AR-co-regulator interaction. A designed molecule or mini-protein can be used to change the conformation of AR in the way it does not recruit that particular co-regulator/s. The inhibition of the complex formation can be later checked by native MS.

Since up regulation of AR activity is likely the cause of prostate cancer, another possible method to treat prostate cancer can be via inhibition of enzyme activity inherits AR modifications such as phosphorylase.

In this report we describe an efficient proteomic analysis for the identification of novel AR complexes in stroma and epithelium of normal and malignant prostate cells.

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1. Introduction

1.1 Nuclear Receptors (NR)

Small hydrophobic metabolites like steroids, retinoic and bile acids, have tremendous effects on cell physiology. They play an important role in the regulation of proliferation, differentiation as well as the cellular metabolism [1-2]. Due to their ability to promote proliferation and/or differentiation small metabolites can also play a crucial role in the dysfunction of cells and hormone dependent tissues [7-9]. Most prominent examples are the pro-proliferative activity of estrogens on breast cancer and of testosterone on prostate cancer [1-3].
In the recent years it became clear that the biological effects of theses compounds are mediated by proteins of the nuclear receptor (NR) superfamily. In human 48 genes have been identified that code for NR [4]. This number varies in other species. These receptors are ligand activated multi-domain transcription factors. They bind to DNA response elements and regulate expression of target genes in response to ligand binding and recruitment of co-regulator proteins [5].
The common modular structure of a NR includes a conserved N-terminal DNA-binding domain (DBD) composed of two zinc fingers, and a C-terminal ligand-binding domain (LBD) which consists of 12 α-helices [2]. The flexible region between DBD an LBD is called Hinge domain (figure 1.1a). In addition, to determining ligand specificity, the LBD contains a ligand-inducible transactivation function 2 (AF-2), which is controlled by the position of helix 12. Upon binding of ligand, NRs change their conformation and some of them translocalize to the nucleus [1-5]. Ligand binding also opens the DNA-binding domain and enables NRs to regulate transcription of their target genes [4]. Additionally the C-terminal α-helix of the LBD (H12, AF-2) folds over to close the ligand binding pocket creating a hydrophobic surface which can be recognized by co-regulator [6].

Historically nuclear receptors have been divided into type I receptors, which undergo translocation to the nucleus upon ligand binding and type II receptors, located in the nucleus at all times [1-2].

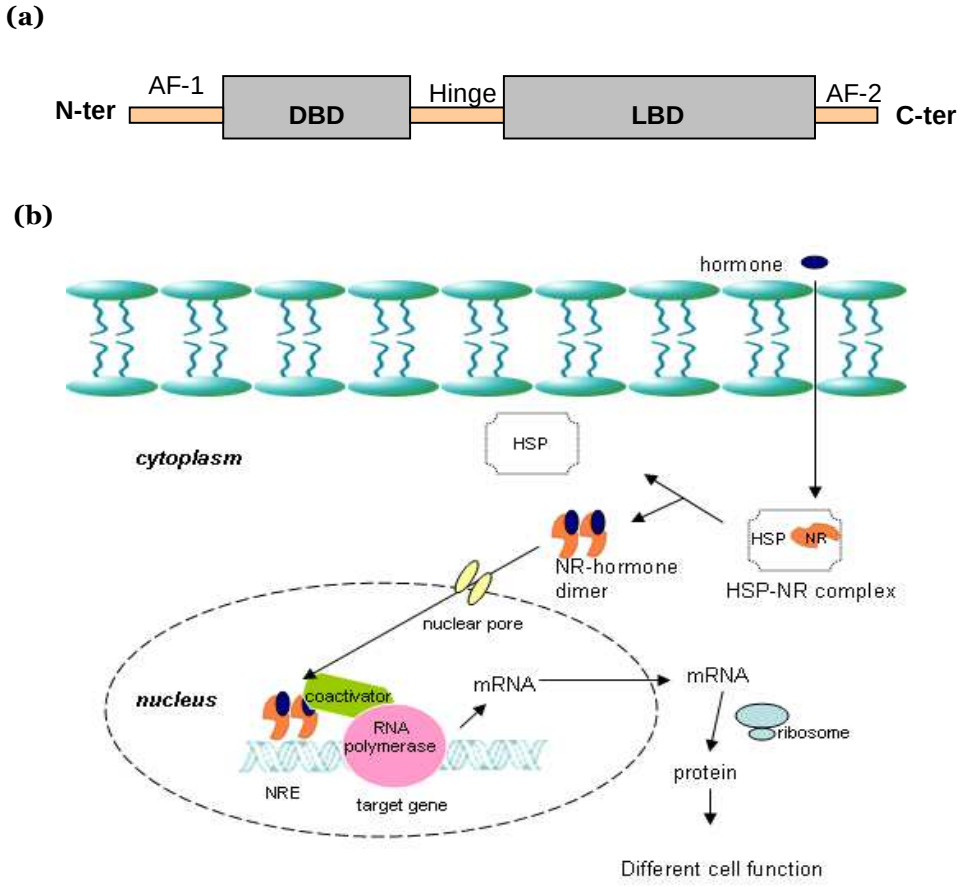


Figure 1.1 a) *Schematic overview of NR's modular domain. AF1 and AF2: transcriptional activation function 1 and 2, respectively, DBD: DNA binding domain, LBD: ligand binding domain.* ***b)*** *Type I NRs pathway. Upon hormone binding the dimerized receptor dissociate from co-repressors in the cytoplasm and translocate into nucleus. After binding to the DNA and recruitment of co-activators they promote chromatin remodeling and activate transcription.*

Type I receptors are localized in the cytoplasm and are protected by heat shock proteins. This complex protects the receptor from denaturation and aggregation. Ligand binding induces dimerization which causes recruitment of different co-regulators. This multi-protein complex regulates gene transcription after binding to specific DNA regions called hormone response elements (HREs) (figure 1.1b).

Type II receptors (TR, RAR, etc.,) remain in the nucleus regardless of the presence of ligand and usually bind to the DNA as heterodimers with retinoid X receptor (RXR) [11]. Furthermore NRs can be divided in subgroups according to their ligands. For instance, the subgroup of the steroid hormone receptors (SHRs) performs signal transduction for steroid hormones, e.g. estradiol (E2).
NR's ligands are lipophilic and pass through the membrane prior to receptor binding [6]. Studies have shown that some of these ligands feature tissue-specific and selective activities. This is crucial for the separation of the effects of NR stimulation or inhibition from undesired site effect which can be regulated by the same receptor. In general NR ligands are mainly classified into four classes [5-6]:

1. Full agonists: promoting co-activator binding
2. Full antagonists: promoting co-repressor binding
3. Selective NR modulators: promoting co-activator and co-repressor binding
4. Inhibitors: Preventing co-activator recruitment

Sequence comparison of the NRs in human shows high variability in LBD explaining the specificity of the signal receptor for a small number of ligands. Bound ligands allosterically influence the position and the orientation of H12, which governs co-regulator binding [8]. When an agonist is bound to the ligand binding pocket, H12 is repositioned compared to the folding state without a ligand. This exposes a functional interface for co-acitvator recruitment via the conserved LxxLL motif (figure 1.2a) in the co-activators [9]. Antagonists instead induce a different conformational change that blocks the binding of co-activators (figure 1.2b).

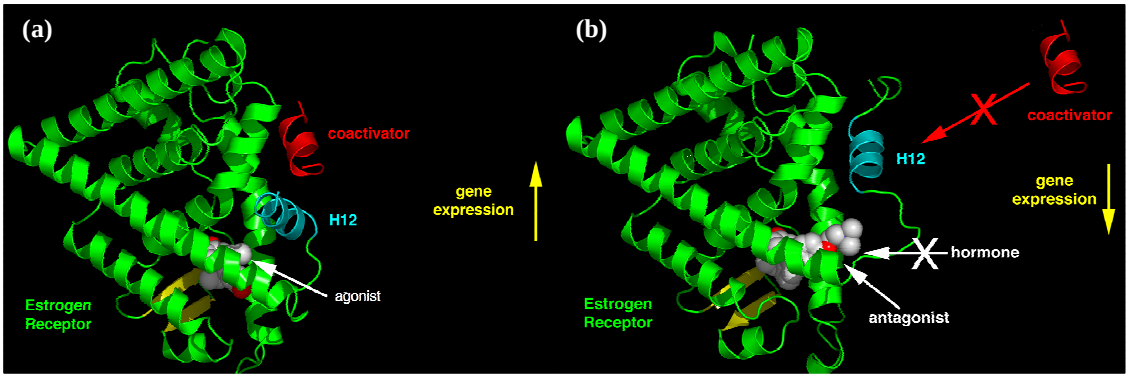


Figure 1.2 *Crystal structure of human ERα-LBD (agonistic and antagonistic conformation). LBD: green, H12: blue, transcriptional intermediary factor 2 (TIF2) with helical LxxLL motif: red,* ***a)*** *in complex with both the physiological ligand 17β-estradiol (agonist) and a co-activator,* ***b)*** *in complex with the antagonist 4-hydroxy-tamoxyfen* [12]

1.2 NR protein complexes

NRs act as ligand-dependent nuclear transcription factors. However, their activity and specificity can also be modulated by co-regulators that are recruited upon ligand binding. There are two types of co-regulators: co-activator and co-repressor (figure 1.1). Co-activators increase and co-repressors decrease transcriptional activity.

The LBD is responsible for ligand recognition and co-regulator interaction of both co-activator and co-repressor. In the absence of a ligand, receptors are often assembled with chaperone proteins in NR-co-repressor complexes. These are located in the cytoplasm resulting in suppression of transcription [12-14]. Co-repressors, on the other hand, seem to bind to a surface that overlaps with the binding region of co-activators and therefore hinder co-activator binding [20]. Some receptors are associated with co-repressors that silence their activity in the absence of ligands. In this case, activation occurs via displacement of the associated co-repressor by a co-activator [21]. However, co-repressors-NR-interaction in the absence of a ligand could not be observed for all receptors. Another possibility is the recruitment of co-repressors upon antagonist binding that induces conformational change of the receptor [9].

Co-activators are usually large proteins with several activation domains. Estrogen-Receptor-associated protein 160 (ERAP160) was the first identified protein that mediates hormone-induced transcription. Steroid receptor co-activator-1 (SRC-1)/RIP160 gene was the first to be cloned as a co-activator of NRs [15-17]. To this day, approximately 300 NR co-regulators have been characterized [8]. Structural analyses of co-activators have shown that many of them have a Leu-Xaa-Xaa-Leu-Leu consensus sequence (Leu, leucine; Xaa, any amino acid) [9,18]. In addition, co-activators might have other functional motifs, such as bromodomains which is crucial for interaction with other transcriptional factors like, p53; and RNA interacting domains. Co-activators also possess enzymatic activities required for NR-mediated gene transcription like acetyl transferase, ubiquitin ligase, phosphokinase, hydrolase, helicase, phosphatase [18-19].

Previous studies indicate that different external signals lead to distinct patterns of post-translational modification on co-activators recruitment [10]. Therefore, each co-regulator can direct a specific cellular function. However, not much is known about the function of the different co-activators in NR mediated transcription.

1.3 Androgen receptor (AR) and co-regulators

Androgen receptor is a member of the homodimeric sub-group of type I steroid receptors. Other members of this group are Progesterone Receptor (PR), Glucocorticoid Receptor (GR), Mineralcorticoid Receptor (MR) and Estrogen Receptor α and β (ERα/ERβ) [1-2].

AR gene is located on chromosome xq12 and it is 180 kb gene consisting of 8 exons. From N- to C-terminus, AR is composed of an N-terminal transcriptional AF-1 domain, a DBD, a short Hinge region and a c-terminal ligand-binding/AF-2 domain (figure 1.3). Binding of ligand to the AR LBD causes conformational change in C-terminus that induces formation of the AF-2 co-activator binding

surface. Furthermore, AR AF1 it is known to be the major domain responsible for mediating AR transcriptional activity [20].

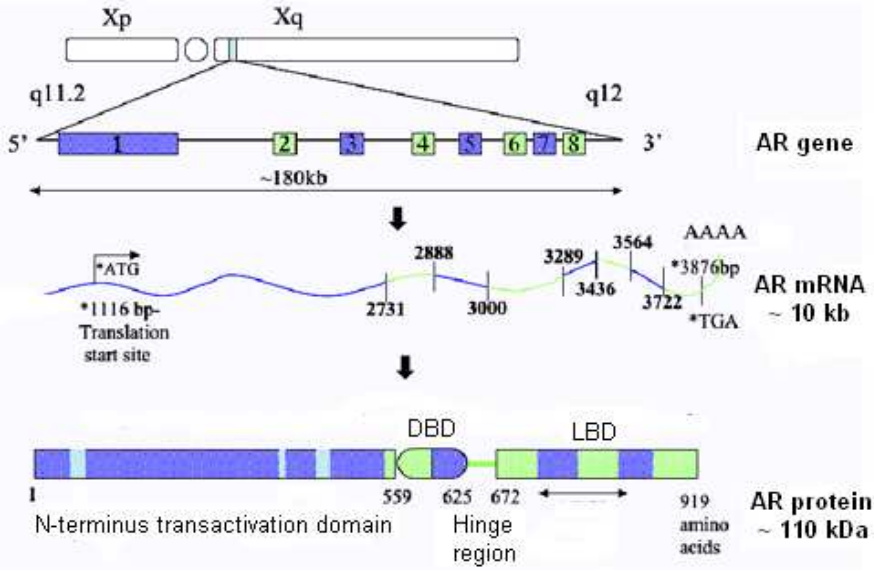


Figure 1.3 *Layout of the AR gene, mRNA and protein with indicated regulatory regions [23]*

AR mediates the cellular action of androgens, the male sex steroids. Androgens are responsible for male sexual differentiation, development and maintenance of sexual tissues in the adult. Additionally, androgens are crucial for the progression and development of age-associated pathologies in men, like prostate cancer and prostatic hyperplasia [22].

The N-terminal domains of this class of receptors are highly divergent in sequence, which is likely to be the bases for differential roles and modes of regulation for these transcription factors (figure 1.4) [11].

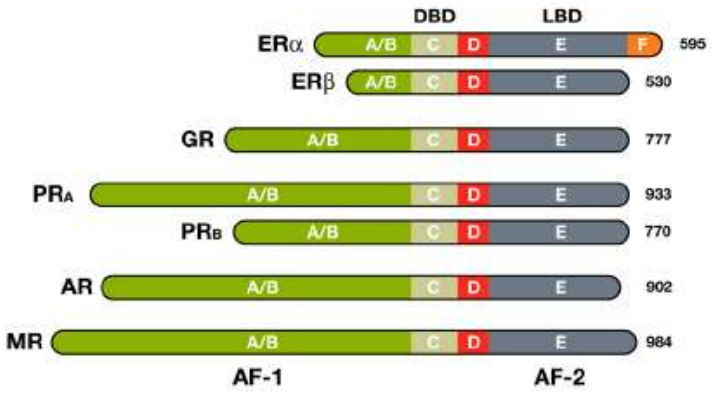


Figure 1.4 *Domain structures of the steroid hormone receptors SHRs. Numbers represent the length of the receptor in amino acids. DBD: DNA binding domain, LBD: ligand binding domain, GR: glucocorticoid receptor, MR: mineralocorticoidreceptor, PR: progesterone receptor* [12]

AR has two physiological ligands, testosterone and dihydrotestosterone (DHT). The unliganded AR is sequestered in the cytoplasm in a complex with heat shock protein (family of chaperones). Whereas dimerized, agonist-bound AR is translocated into the nucleus and engages with androgen response elements (AREs) in the promoter and enhancer regions of genes that are important for growth and survival of normal and cancerous prostate cells [24-25]. It is important to mention that the AR independent of its role as a transcription factor mediates crucial cellular function in the cytoplasm [26]. As an example, studies have shown that the AR participates in rapid signaling cascades, through association with the c-Src tyrosine kinase [27].

Binding of the ligand to AR causes dissociation of the protein from heat-shock complex and translocation of AR into nucleus, then remodeling of chromatin structure at target promoters, recruitment of the transcription machinery and RNA polymerase activity [28].

All these events are modulated by co-regulators that can promote (co-activators) or inhibit (co-repressors) AR function. In general these regulator proteins are divided into 4 major groups [13]:

1) Heat-shock proteins that mediate AR proper folding and stability and movement. Hsp70, Hsp40, hop, Hsp90 and p23 is the minimal chaperone complex required for AR function

2) Histone modifiers. Like, CBP/p300, NCoR2
3) Transcription complex. for example, TRAP/DRIP/ARC
4) DNA structural modifiers. Like, SWI/SNF/BRG1

Table 1.1 *List of identified AR-Co-regulators and AR binding domains* [13]

Co-regulator	Binding site on AR (aa)
ARA24/RAN	11–209 [97]
STUB1/CHIP	220–270 [98]
NCOR2/SMRT	171–328 [99]
ART-27/UXT	153–336 [100]
TF11F	141–485 [101, 102]
Zimp10	1–333 [103]
CBP	1–566 [104, 105]
AES	1–559 [106]
TFIIH	38–561 [107]
STAT3	234–558 [108]
BRCA1	1–555 [109, 110]
DAXX	1–560, 486–651 [111]
BRCA2	1–556, 668–919 [112]
SRC1	360–494, 625–919 [113]
GSN	486–651, 666–918 [114]
Rb	502–565 [115]
PIAS1	544–634 [116, 117]
p300	550–663 [118]
ARIP4	544–623 [119]
ARIP3/PIASXa	554–644 [120]
ARA55/HIC-5	595–918 [121]
TRAP220	622–919 [122]
ARA70/NCOA4	624–919 [44]
TIP60	625–919 [123]
TIF2/GRIP1/SRC2	644–919 [124]
ARA54	653–919 [125]
AIB3/ASC-2	675–919 [126, 127]
CYCLIN D1	1–502, 633–668 [128]
ARA160/TMF1	38–643, 553–918 [129]
NCOR1	1–500, 661–919 [130]
AIB1/SRC3/ NCOA3	1–660, 624–919 [131]

Table 1.1 represents the identified co-regulators and their corresponding AR interaction domains that have been reported so far [13].
Many of the AR co-regulators were identified by *in vitro* experiment of physical interaction-dependent yeast two hybrid screens [13]. However, many more might exist that cannot be identified in *in vitro* experiments.
So far, the precise role of co-regulators in mediating AR function in different state in growth, aging, development and diseases is poorly understood. Most authors assume that recruitment of co-activators is highly regulated [22,24,25]. Unfortunately, current *in vitro* experiments like yeast-two hybrid experiments can not comprehend the complexity of specific and regulated co-activator recruitment.
Additionally, studies have shown that post-translational modifications -for example phosphorylation of AR- also play an important role in the transcriptional activity of the receptors [10,29]. This is probably due to altering the recruitment of co-activators.

1.4 Prostate cancer

Development of cancer in the prostate is one of the most prevalent types of cancer in men over the age of fifty [30]. It is known that in prostate cancer the cells of the prostate glands transform into cancer cells. The prostate tissue consists mainly of stroma and epithelial (glandular) cells. The majority of cancers drive from the epithelial cells while stroma cancer is rare. Both cell types express AR and respond to androgens [31-36]. Androgen, the ligand for AR, includes testosterone, dehydroepiandrosterone and dihydrotestosterone which are made in the testes, adrenal glands and from conversion of testosterone in the prostate respectively. Both, ARs in the stroma as well as the one in the epithelium respond to androgens. However, studies investigation normal prostatic development have established that stromal, but not epithelial AR, is essential for specification of epithelial cell identity, ductal branching, proliferation and

apoptosis [37]. In contrast, in the majority of prostate cancers epithelial AR increases cellular proliferation. Studies on genetically modified mice have shown that loss of AR in the stroma reduces proliferation of the epithelium while loss of AR in the epithelium causes de-differentiation and increases proliferation [37]. Currently there is no satisfactory explanation for the paradox effect of the same receptor in two different cell types but most scientist assume that differential recruitment plays an important role.

Due to the pro-proliferative activity of AR, androgen deprivation therapy is one of the state of the art treatments of prostate cancer. However, after many years of androgen deprivation therapy many patients become resistant and develop androgen independent cancer [38,39]. The exact mechanism of resistance is unknown.

In many prostate cancers and prostate cancer cells the up-regulation of androgen receptor and overexpression of a certain cofactor, mainly co-activator, have been reported [40,41]. For example CBP is highly expressed in advanced prostate cancer and also in tissues from patients that failed therapy [41]. The incidence of mutation in the AR in carcinoma cells ranges from 20 to 40% and over 60 mutations have been identified [42,43]. The mutations frequency in primary prostate cancer is low whereas they can be detected with greater frequency in metastatic tumor cells. Interestingly, the majority of AR point mutations identified in prostate cancer map to 3 regions, the LBD (aa 670-678, the boundry between LBD and hinge), loop between helices 3 and 4 of LBD (aa 701-730, involve in ligand recognition and specificity) and the area flanking AF-2 (aa 874 to 910, a region involved in co-regulator binding) [44,45].

Moreover, different mutations in distinct co-regulators have been observed in tumor cells. That indicates AR co-regulators are important regulators of epithelial cell fate and incorrect recruitment of co-regulators might promote progression of cancer.

1.5 Role of co-regulators in AR transactivation and prostate cancer

There is one important question that needs to be answered to understand the role of AR in the prostate: Is the different role of AR in distinct cell line, eithelial and stroma, due to different co-regulators recruitment?

It was shown previously that p160 co-activators are the most understood complex of these co-regulators. This complex consists of steroid receptor co-activator 1 (SRC1), transcriptional intermediary factor 2 (TIF2) and glucocorticoid receptor interacting protein 1 (GRIP1) [46-50]. Their recruitment by ligand bound AR directly influences AR transactivation capacity via histone acetyltransferase activity [51]. They act indirectly as a platform for the secondary co-activators recruitment of acetyltransferase such as CBP/p300 [52] and pCAF [53] or protein methyltransferase like CARM1 or PRMT1 [54,55]. CBP/p300 contributes to transcriptional activation by remodeling chromatin and the recruitment of the basal transcription factors (TFIIB and TBP) [54,55,56]. Subsequently, nucleosome remodeling complexes (like SWI/SNF) are recruited followed by mediator complexes [57]. The mediator complexes connect DNA-bound transcription factors with the general transcriptional machinery such as RNA polymerase II [58]. Due to chromatin remodeling activity these co-regulators can influence the nature of genes that are regulated by AR and hence determine the fate of the cell. Immunohistochemical evaluation of the p160 co-activators in prostate cancer tissues shows over expression of SRC1 in half of the cases [59]. Furthermore, up regulation of p300 can be used to predict extra-prostatic extension. It has been also shown that level of CBP is highly expressed in advanced prostate cancer [40,41]. Another example is ARA55 which is an AR co-activator that preferentially expressed in prostate stromal cells [60]. This results in increased AR activity and altered specificity of receptor binding to alternate ligands. The majority of studies have reported down regulation of mRNA of ARA55 in malignant prostate tissue in comparative with non-malignant [61-62].

Beside co-activators, co-repressors are the other AR co-regulator complexes. Recruitment of the co-repressors is believed to regulate antagonist mediated inhibition of AR [24,63]. Therefore it is possible that loss of expression of co-repressors could facilitate tumor growth [64-66]. Two well known AR co-repressors are the nuclear receptor co-repressor (NCoR1) and the silencing mediator of retinoid and thyroid hormone receptor (SMRT/NCoR2) [30]. According to the studies the interaction of SMRT and NCoR1 to agonist and antagonist bound AR suppress agonist-induced activation of androgen regulated genes [67]. Since the co-repressors compete for the same AR binding surface as co-activators, their expression level is a crucial factor for determination of the level and consequences of AR signaling in prostate cancer cells.

Different studies have established variation of co-regulators expression in different stage of prostate cancer development [30,63-67]. In general the transactivation potential of AR derives from the capacity of ligands to induce the proper formation of AF-2 surface within the LBD which is important for recruitment of FxxLF motifs found in multiple co-regulators [68,69]. Although the consequences of this competition in not clear yet, shifting AR structure influences composition of the transcription complex and target gene selectivity and specificity.

This data raising the question: is it possible that one cause of the failure of long term androgen deprivation therapy is in the constant recruitment of a yet unknown co-activator? And finally, can co-regulators be used as targets for the treatment of prostate cancer?

1.6 Research aim and hypothesis

The major intention of this study is to analysis the AR complexes in epithelial and stromal cells of prostate cancer and normal tissue.

Therefore this paper presents a work flow that can lead to the discovery of novel *in vivo* co-activators and co-repressors. Additionally the techniques described in

this paper can help to identify post-translational modifications of AR and co-activators that might be important for AR activity.

2. Experimental approach

2.1 Proteomics

Proteomics is a technique to identify proteins and protein modifications on a high throughput scale. The proteomics work flow consists of initial separation steps of proteins, then the digestion of the separated proteins with a well characterized protease, like trypsin [70]. Most tryptic peptides have to optimal length and chemical properties for further separation and analysis. The generated peptides are then further separated using high pressure liquid chromatography (HPLC) and analyzed by mass spectrometry (MS) [70]. Eventually, protein identification is performed with the database the assigns proteins to the identified peptide sequences. The crucial step in the workflow is the MS analysis. MS is an analytical method to determine the mass and composition of an ionized compound. MS therefore measures the relative velocity or deflection of a charged molecule in an electrical field in the high vacuum. From this data one can calculate the mass and charge of the peptide. Two ionization techniques have been developed for the ionization of macromolecules, including proteins and peptides: electrospray ionization (ESI) and matrix-assisted laser desroption/ionization (MALDI) [71,72].

Ionized peptides can then be analyzed with various methods. Today ion traps are the most common peptide analyzers [70-72]. Peptides can be additionally fragmented in order to obtain sequence information. Therefore, additional energy is applied to the peptide so that its backbone breaks. Breakage of the backbone results in specific fragments that indicate the initial sequence [70,80,81]. The mass and the sequence data are then searched against databases such as SwissProt to obtain potential identifications (figure 2.1). With this approach, several thousand proteins can be identified in a single LC run. Moreover, sequence information combined with the mass of the parent peptide ion mass allows the identifications of post-translational modifications.

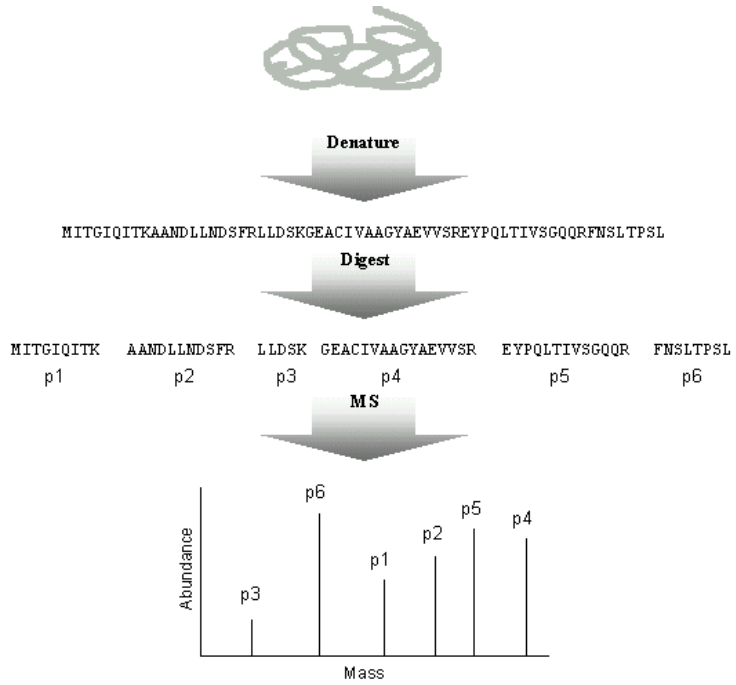


Figure 2.1 *Schematic work flow of a protein digestion and the resulting MS spectra. The peptides p1 to p5 have distinct masses that are determined by their sequences. In a mass spectrometer this masses can be separated and measured. From the initial peptide mass and the additional sequence data (MS/MS) the original protein can be identified..*

2.2 Identification of AR co-regulators

We think that the different activities of AR in different cells are due to the recruitment of different co-regulators. To test this hypothesis we would like to identify the proteins that are associated with AR in stroma, epithelium of normal and malignant prostate. One of the well established biochemical methods for identification of novel protein complexes is affinity purification assay [73]. In this technique endogenous AR complexes from cell can be trapped on short DNA stretches (ARE) that are immobilized on beads. Subsequently by loading the different cell lysates the endogenous AR in complex with its co-regulators can be arrested [73].

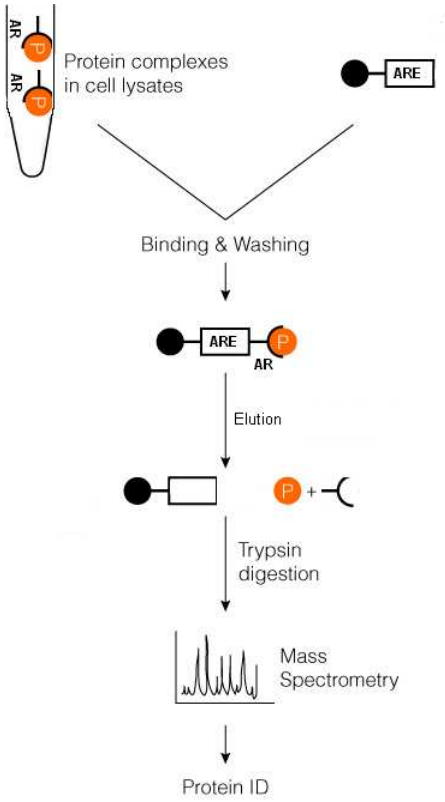
To study different AR co-regulators in prostate cancer development six different cell types will be used:

- Normal prostate epithelial cells
- Normal prostate stroma cells
- Primary prostate tumor epithelial cells
- Primary prostate tumor stroma cells
- Metastatic prostate epithelial cells
- Metastatic prostate stroma cells

The lysates of the above mentioned cells will be loaded onto the beads. The beads contain immobilized ARE. AR in complex with co-regulators will bind to ARE and be captured on the beads. The bound proteins will be washed several times to eliminate unspecific bound proteins in the complex. Subsequently, the AR-co-regulators can be eluted by changing the pH of the solution. In parallel, a negative experiment can be performed by beads immobilized with non-sense DNA - for instance the same nucleotides but in a random order.

The Next step will be separation of the proteins bound to AR on gel electrophoresis. From the gel, the proteins will be excised and digested with trypsin. Then the tryptic peptides will be separated by Liquid chromatography (LC) and analyzed by MS. From the fragmentation patterns of the peptides the sequences can be read and eventually the protein ID will be extracted (figure 2.2). One of the disadvantages of this technique is the high number of false positives and negatives [70]. That means proteins might be identified in the complex *in vitro* experiment eventhough they are not associated *in vivo*. To determine the specificity of a protein for the AR complexes the eluted proteins from the scrambled DNA (negative control) can be labeled with a tag that contains only heavy isotopes of an element, like ^{13}C. The same tag containing only light isotopes will be used in the positive experiment. After mixing the eluted proteins from negative control and AR complex purification they can be analyzed by MS. MS analysis allows the discrimination of tags (according to their masses) and subsequently the quantification of both species. Since we expect specific binding partners to be more abundant in the sense ARE experiment, we can use this data to eliminate false-positiv proteins. By analyzing and comparing the MS data from different cell type we will eventually be able to recognize the differences in AR-co-regulator recruitment in different stage of the prostate cancer.

Figure 2.2 *Experimental procedure. AR complexes of the cell lysate are immobilized on the ARE beads that contain bound ARE. Then the protein complexes are eluted, digested with trypsin and analyzed by MS..*



2.3 Study post-translational modification of AR

After identification of the different co-regulators associated to AR in epithelial and stroma cells of normal and malignant prostate, the next step will be the evaluation of the members of these complexes.

We expect to identify different complexes of co-regulators for the same receptor in different cell types. However, how can this be explained considering that in all cell types the ligand is testosterone or dihydrotestosterone? We assume that recruitment of co-regulators is regulated not only the three-dimensional structure of the receptor itself but also by modifications of the protein.

More and more studies document that AR can be modified at various positions [10] and these modifications play an important role in transcriptional activity [10,13,22]. Therefore AR from the immunoprecipitation of normal and malignant

prostate epithelial and stroma cell lysates will be searched for potential modification sites by MS. In the future this data might help to identify post-translation modifications that can alter co-activators recruitment.

2.4 Evaluation of co-regulators

One technique to evaluate the role of a protein in transcription is luciferase assays [74]. In this technique androgen response elements (ARE) can be cloned up stream of firefly luciferase gene. Then cells are transfected with this construct, a plasmid that contains the coding region of AR and the potential co-regulator. In the presence of co-activators ligand bound AR recognizes ARE and the firefly luciferace gene is transcribed. In the present of AR-co-repressor complex low signal of firefly luciferace gene will be observed using luminescence detector. Secondly, native MS will be performed with AR in the presence of different ligands and potential co-activators. Therefore this technique can be used to measure the stechiometry of AR complexes in the presence of different ligands.

2.5 Identification of modified sites of AR co-regulators

Recent studies have indicated that multiple post-translational modifications can affect AR activity [10,13,22]. They stabilize protein for the fine-tuning of gene transcription event; modulate AR-co-regulators interaction and sub-cellular localization [10]. Moreover, also co-regulators can be modified which additionally alter their function [29]. For example phosphorylation of co-regulators can increase recruitment of other co-regulators and members of the transcription machinery [75,76,77]. Post-tranlastional modification of AR co-regulators from distinct cell types can be identified by MS. The date obtained from session 2.2 and 2.3 will also be searched for possible modifications sites after separation of the protein complexes on gel by MS.

2.6 Investigate AR co-regulators' structure with native MS

The principle of MS involves in ionizing a molecule then measuring its mass-to-charge ratio and using the obtained ratio to indicate molecular weight. This process occurs in a vacuum system with electric fields, therefore proteins are usually denatured. However, under careful conditions, the proteins can be kept in their native state. Interestingly, not only single protein can be in its native condition, but complexes of multiple molecules can be kept together [78]. By measuring the weight of these complexes and their individual component the quaternary protein structures can be investigated. So, by utilizing native MS we can identify ligand-AR-co-regulator complexes and the topology and dynamics of these complexes.

We should take in our account that the most important requirement for choosing a mass analyzer when using native MS is its ability to precisely identify ions with high mass over charge (m/z) values. So far in this field the leading mass analyzers are orthogonal ESI-time of flight (ToF) or Q-Tof apparatuses. Furthermore a quadrupole mass filter that is adjusted to lower frequencies will be used to allow for the transmission and mass analysis of ions with high m/z values [78]. What is also important to mention about native MS is that by increasing pressures in quadrupole instruments the desolvation and transmission of very large and slow ions can be optimized.

In the present study we will use this technique to answer a crucial question: is the recruitment of certain co-regulators dependent on the structure of the ligand? Therefore purified AR will be treated with different ligands in the presence of the previous identified co-activators. Then the mass of the protein complexes will be measured by native MS. From the total mass and the mass of the monomeric subunits we will determine the stechiometry of the complex.

3. Discussion

3.1 Regulation of AR co-regulators in prostate cancer

The formation of an active AR-directed pre-initiation complex normally occurs via the sequential recruitment of co-regulators with different functions to the ligand bound nuclear AR [30]. The AR signaling has a critical role in prostate development but also in the development of prostate cancer. Many of the putative AR co-regulators were identified using physical interaction-dependent yeast-two-hybrid screens with discrete AR receptor domains as bait [13], but there is a paucity of data concerning their ability to influence AR regulated gene-specific expression. Moreover, yeast-two-hybrid screens do not allow the discrimination of the transcriptional active and the quiescent complex. Neither it is possible to distinguish the complexes in different cell types and at different stages of development. Therefore additional studies are necessary to identify the members of the active complexes in their endogenous environment.

The suggested proteomic analysis of DNA-bound AR complexes in stroma and epithelium of normal and malignant prostate is likely possible since previous studies were able to performed very similar experiments with different proteins like ERα [79-85]. Moreover, using different ligands the authors could show that indeed ERα recruits different co-regulators to the active complex [85]. Hence this technology can provide the data that are necessary to understand complex formation in different environments. The approach targets the active complex in its endogenous environment. Trapping nuclear receptor complexes with their endogenous elements however holds several experimental problems. In general the amount of protein that can be isolated with this approach is low and therefore protein identification can be limited. That can be solved by either increasing the amount of the start material or improving MS sensitivity. The sensitivity of MS machines has increased dramatically in the last few years. Several studies have shown that the current state of the art mass spectrometers can identify proteins

in the femtomolar range. Therefore mass spectrometry is not longer the limiting factor. The improved sensitivity on the other hand results in a high number of false-positive identifications. A protein can be false-positively identified because it unspecifically binds DNA or because of its high abundance. In the recent years several different isotope-labeling strategies for proteomics have been developed. They allow the relative quantification of proteins in complex samples. Isotope-labeling has also been used to distinguish between false positive proteins in complexes. In this approach proteins that appear with the same intensity in the negative control experiment are eliminated. Therefore, state of the art mass spectrometry in combination with isotope-labeling allows the identification of co-regulators in nuclear receptor complexes.

In a second set experiment it is necessary to evaluate which of the identified proteins can modulate the transcriptional activity of the receptor. For instance, identified co-activators from proteomic experiments can be knockdown and overexpressed one by one in normal cells. The phenotype of the cells will indicate whether the particular co-activator is involved in proliferation or differentiation of the cells. Moreover the transcriptional activity of AR complexes in these cells can be measured. This data will provides a list of proteins that can modulate AR activity in normal and cancerous cells and eventually might lead to the identification of a novel co-activator that plays an important role in prostate cancer

3.2 Role of PTM in AR co-regulators interaction

Studies have shown that one way of modulating the AR complex is by altering post-translational modifications of AR [29,75-77]. For example acetylation of AR not only regulates assembly of the transcription complex through co-activator/co-repressor complex binding, but also modulates receptor transactivation by other signaling pathways.

To determine the role of PTM in AR co-regulators interaction it is important to map PTMs in the active complex. The knowledge of regulatory modification sites is the first step in the search for the enzymes that introduce them. Once these enzymes are discovered one can aim to inhibit the activity of the enzyme which in turn results in blocking of particular modification.
Last but not least formation of AR complexes in the presence of different ligands and co-regulators can be studies by native MS. This is of particular interest for the development of new synthetic ligands for AR. Synthetic antagonists for ERs have been successfully introduced in the clinics and are used to fight breast cancer since many years [86-88]. In prostate cancer similar antagonists are missing. Once important co-activators of AR have been identified, ligands that prevent binding of these co-activators to the complex can be developed. The potential of a ligand to block co-activator recruitment can be evaluated by measuring protein complex masses in the presence of the new ligand and the co-activator.

3.3 Possible strategies to treat prostate cancer

Uncontrolled proliferation is one of the hallmarks of cancer. In the prostate AR is one of the central proteins that regulate proliferation. Hence traditional prostate cancer hormonal therapy aims at silencing AR signaling. However, prostate cancer progression is also marked by the gradual loss of differentiation of the epithelium. A low state of differentiation is an indicator of poor prognosis. However, currently there is no medication that can improve the differentiation status of the epithelium.
Recent studies on transgenic mice indicated that AR under certain circumstances can also promote differentiation. Unfortunately the co-factors that are necessary to shift AR activity from promoting proliferation to promoting differentiation are unknown. It is very tempting to speculate that once these factors are identified novel agonists can be developed that promote their recruitment. These agonists would provide an entirely new strategy to tackle prostate cancer.

Since up regulation of AR activity is likely the cause of prostate cancer [45], another possible method to treat prostate cancer can be via inhibition of enzyme activity inherits AR modifications such as phosphorylase [76].
Today androgen withdrawal is still the therapy of choice for androgen dependent prostate cancer. However, most patients will be insensitive to this treatment within a couple of years. How this happens is unknown. Most likely AR becomes constitutively active by an unknown mechanism. By identifying and characterizing AR-co-regulators complexes we might be able to identify a novel target the inhibit AR by activating a co-repressor.

3.4 Summary

In this paper we propose a proteomics based strategy to identify endogenous AR co-regulators. Our technique allows the discrimination between co-regulators in different cells and states of development including cancer and so answer one of the most puzzling questions in endocrinology: How can one receptor in the presence of the same ligand and the same response element have such divers effects in different cells?

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