SATU PASSINEN

Hsp90 in Progesterone Receptor Action

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ACADEMIC DISSERTATION

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to Veikko Passinen
and
Matti Räsänen
and
my family

ABSTRACT

In mammals the gonads and adrenal glands produce five major groups of steroid hormones: androgens, estrogens, glucocorticoids, progestins and mineralocorticoids. They all regulate a large number of physiological processes in the target cells equipped with the corresponding steroid hormone receptors. Steroid receptors belong to the nuclear receptor superfamily and function as ligand regulated transcription factors. All steroid receptors are modular proteins composed of distinct regions A-F. The intracellular distribution of steroid receptors is the result of ATP-dependent cytoplasmic-nuclear shuttling and passive diffusion from nucleus into the cytoplasm. Steroid receptors have NLSs (nuclear localization signals) and they are constantly moving into and out of the nuclei. The localization of steroid receptors is not static. First in vitro studies proposed that unliganded steroid receptors locate in the cytoplasm and translocate into the nucleus after binding hormone. Immunohistochemical studies have concluded that steroid receptors are located in the nucleus independent of the hormonal status.

All unliganded steroid receptors are associated with multiprotein complex of chaperones which maintains the receptor in an inactive state but keeps it prepared for hormone binding. Chaperones are proteins that are responsible for maintaining the correct folding, function and stability of client proteins. The chaperone facilitates, for example, oligomer assembly, transport to a particular subcellular compartment and controls the conformation of the receptor. Steroid receptors can be seen as a hetero oligomeric complex with several proteins in vitro in cell homogenates.

Hsp90 (Heat shock protein 90) was the first protein known to interact with steroid receptor. It accounts for 1-2% of all cellular proteins in most cells. It is known to interact in a ligand dependent manner with the ligand binding domain of the steroid receptor. Most immunohistochemical studies have shown that Hsp90 is located in the cytoplasm. However there are studies showing that Hsp90 is located in the cytoplasm and nucleus. Hsp90 does not have NLS. In vitro studies have shown that Hsp90 is a chaperone.

We wanted to study the oligomeric complex between Hsp90 and steroid receptors seen in in vitro cell extract existing in intact cells. We also studied the involvement of Hsp90 in the nuclear transport of progesterone receptor and investigated the determinants of the cytoplasmic location of Hsp90 and the strength of the cytoplasmic signal by comparing it with that of the nuclear transport signals of steroid receptors.In our studies association of steroid receptors and Hsp90 in vivo was studied by using antibody αD which can distinguish between the oligomeric and dissociated forms. Our findings suggest that a portion of the cytoplasmic receptor is seen as an oligomeric complex but in the nucleus most receptors are in dissociated form. The complexes seen in cell extracts might be formed during cell fractionation. We studied the sequences responsible for cytoplasmic localization by constructing hybrid molecules with Hsp90 sequences and progesterone receptor. The strength of the cytoplasmic signal was studied by contructing chimeric molecules between C-terminal half of Hsp90 and estrogen receptor with different numbers of proto-NLSs. The results suggest that Hsp90 contains a cytoplasmic localization sequence. The involvement of Hsp90 in nuclear transport of progesterone receptor was studied by using digitonin permeabilized cells and purified nuclei in vitro transport systems. We could demonstrate that unliganded progesterone receptor which was salt dissociated from Hsp90 was transported into the nucleus whereas Hsp90 remained in the cytosol. When the oligomeric complex of the progesterone receptor was stabilized with molybdate no nuclear accumulation was seen. Molybdate was added after ligand progesterone receptor was accumulated into the nucleus. Our results suggest that Hsp90 assists the chaperoning process of PR preceding nuclear transport of the receptor and the oligomeric complex is short-lived dissociating before or during nuclear transport.

CONTENTS

ABBREVIATIONS

AF	activation function
AP-1	activator protein 1
AR	androgen receptor
ARA70	androgen receptor-associated protein 70
ATG	initiation codon
ATP	adenosine triphosphate
B10	monoclonal antibody to human estrogen receptor
BSA	bovine serum albumin
CBP	cAMP-response element-binding protein
cPR	chicken progesterone receptor
Crm1	member of the importin-beta protein family
C-terminal	carboxyterminal
DBD	DNA binding domain
dl	maternal morphogen dorsal
DMEM	Dulbecco`s modified Eagle medium
DNA	deoxyribonucleic acid
DnaJ	bacterial homolog of Hsp40
αD	polyclonal PR-antibody
EGS	ethylene glycobis(succinimidylsuccinate)
ER	estrogen receptor
ERα	α form of estrogen receptor
ERβ	β form of estrogen receptor
FKBP51	immunophilin
GFP	green fluorescent protein
GR	glucocorticoid receptor
GTP	guanosine triphosphate
GUK	guanylate kinase-homologous region
HAT	histone acetyltransferase
HBD	hormone binding domain
HDAC	histone deacetylase
hER	human estrogen receptor
HOOK	domain of MAGUK
Hop	Hsp-organizing protein
Hsp	heat shock protein
IgG	immunoglobulinG
kD	kilodalton
LBD	ligand binding domain
MAGUK	membrane-associated gyanylate kinase
MAPK	mitogen-activated protein kinase
MR	mineralocorticoid receptor
NCOR	nuclear receptor corepressor
NES	nuclear export signal
NLS	nuclear localization signal
NPC	nuclear pore complex
NR	nuclear receptor
N-terminal	aminoterminal

PBS	phosphate buffered saline
p/CAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PKA	cAMP-dependent protein kinase
PPIase	peptidypropyl isomerase domain
PR	progesterone receptor
PR-A	A-form of progesterone receptor
PR-B	B-form of progesterone receptor
PR6	monoclonal antibody to PR
PR22	monoclonal antibody to PR
RAC3	receptor associated coactivator 3
Ran	a small GTP-binding protein
RAP	receptor associated protein
RAR	retinoic acid receptor
RID	receptor-interacting domain
RNA	ribonucleic acid
RT	room temperature
RXR	retinoid X receptor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMRT	silencing mediator of retinoid and thyroid receptors
SRC-1	steroid receptor coactivator 1
Stat	signal transducers and activators of transcription
Sti1	yeast homolog of Hop
SWI/SNF	switch/sucrose nonfermentable family
TAF	tightly associated factor
TBP	TATA box binding protein
TFIIA	basal transcription factor IIA
TIF2	transcriptional intermediary factor 2
TPR	tetratricopeptide repeat
TR	thyroid hormone receptor
VDR	vitamin D receptor
wt	wild type
Ydj1	yeast homolog of Hsp40
7Dα	monoclonal Hsp90-antibody

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles, referred to in the text by their Roman numerals:

I Passinen S, Haverinen M, Pekki A, Rauta J, Paranko J, Syvälä H, Tuohimaa P and Ylikomi T (1999): Only a small portion of the cytoplasmic progesterone receptor is associated with Hsp90 in vivo. Journal of Cellular Biochemistry 74:458-467

II Haverinen M, Passinen S, Syvälä H, Pasanen S, Manninen T, Tuohimaa P and Ylikomi T (2001): Heat shock protein 90 and the nuclear transport of progesterone receptor. Cell Stress and Chaperones 6(3):256-262

III Passinen S, Valkila J, Manninen T, Syvälä H and Ylikomi T (2001): The C-terminal half of Hsp90 is responsible for its cytoplasmic localization. European Journal of Biochemistry 268:5337-5342

IV Passinen S and Ylikomi T (2003): Evidence for the existence of an oligomeric, non-DNA-binding complex of the progesterone receptor in the cytoplasm. European Journal of Histochemistry 47(3):201-208

INTRODUCTION

Steroid hormones control various cellular activities. Sex steroids induce development and differentiation of the reproductive system, musclinization and feminization, control reproduction and reproductive behavior in adults. The action of steroids is mediated by specific intracellular receptors. The receptors consist of similar molecular structures and form a nuclear receptor superfamily. Nuclear receptors are transcription factors and are regulated by ligand binding (Laudet and Gronemeyer 2002).

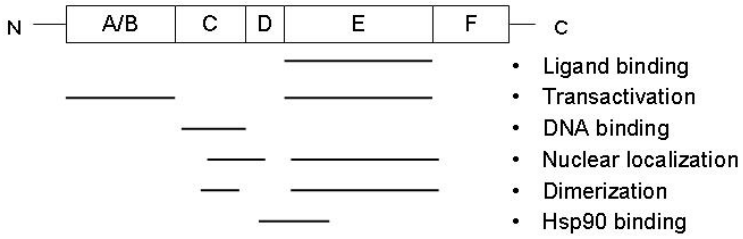
Cell fractionation studies proposed that unliganded steroid hormone receptor translocates into the nucleus after binding hormone (Jensen et al. 1968). Immunohistochemical studies have shown that unliganded estrogen and progesterone receptors are localized in the nucleus of the target cells (Syvala et al. 1996, Hiroi et al. 1999). The entry of the nuclear receptor into the nucleus requires nuclear localization signals (NLSs) and ATP. The localization of steroid hormone receptors is not static and receptors are constantly shuttling between the nucleus and cytoplasm through the nuclear pore complex (Guiochon-Mantel et al. 1996).
Heat shock proteins are a highly conserved group of proteins and are expressed from bacteria to eukaryotes. Their expression is enhanced by increased temperature, metabolic poisons, toxins and response to stress (Welch 1992). Heat shock proteins belong to the chaperoning proteins (Garrido et al. 2001). Chaperones are proteins which bind to and stabilize unstable proteins and facilitate protein`s folding, oligomeric assembly and transport and turnover (Ellis and van der Vies 1991).
Hsp90 is an essential stress protein. It controls the activity, turnover and trafficking of various proteins. Most of the Hsp90 regulated proteins are involved in signal transduction. Hsp90 binds to unliganded steroid receptors and composes 8-9S oligomers in vitro. Steroid receptors are brought into the complex with Hsp90 by heterocomplex assembly that the steroid-binding form is generated by. In reticulocyte lysate receptor-Hsp90 assembly minimally requires Hsp90, Hsp70, Hop, Hsp40 and p23 (Pratt and Toft 2003). There is evidence that receptor-associated proteins are involved in the nuclear translocation of nuclear proteins (Pratt et al. 2004a). The localization of heat shock protein 90 in the cell has been claimed. There are studies that Hsp90 is exclusively in the cytoplasm but there are also reports of cytoplasmic and nuclear distribution of Hsp90 (Gasc et al. 1984, Gasc et al. 1990, Perdew et al. 1993). There is also controversy whether Hsp90 does not form complexes with steroid receptors in the nucleus (Pekki et al. 1994).
This study was undertaken to study the determinants of the cytoplasmic location of Hsp90 and the strength of the cytoplasmic signal. We also wanted to ascertain whether Hsp90 and PR are associated if they are located in the same subcellular compartment in vivo and stabilizing the complex by stabilizing reagents and to observe the nuclear transport of the oligomeric complex and heat shock protein 90.

REVIEW OF THE LITERATURE

1. NUCLEAR RECEPTOR SUPERFAMILY

1.1. Structure of the nuclear receptors

Biological responses to steroid hormones are mediated through their intracellular receptors. The nuclear receptor (NR) superfamily consists of three major categories: 1) steroid hormone receptors 2) thyroid, retinoid, vitamin D and peroxisome proliferator activated receptors 3) orphan receptors (Laudet and Gronemeyer 2002, McKenna and O'Malley 2002). These receptors have a common functional domain structure. Nuclear receptors have six domains (A-F domains), encoded by 8-9 exons. The domains can also be divided into three major functional domains: 1) N-terminal (A, B domain) 2) DNA binding domain, DBD (C domain) 3) ligand binding domain, LBD (E domain). F domain is important in distinguishing estrogen agonists and antagonists. The hinge region (D domain) is a 40-50 amino acid sequence and separates DBD and LBD. It contains sequences for receptor dimerization and nuclear localization sequences (NLSs) (Kumar et al. 1987, Beato and Klug 2000). See Figure 1.



- Figure1. Structural organization of the nuclear receptors and listing of functional activities of the respective domains.

Ligand binding domain (LBD) exists in the carboxy-terminal end of the molecule. The ligand binding domain functions as a ligand binding site and homodimerization domain and regulates receptor activation. The LBD surface alters when it binds agonist or antagonist. Thus the altered surface changes protein:protein interaction and coactivator/corepressor contacts. The LBD binds heat shock and other proteins in a pre-activation complex (Kumar and Thompson 1999). The C-terminal part of LBDs has been shown to have a ligand-inducible activation function AF-2 (Green and Chambon 1988, Nagpal et al. 1993). Ligand binding activates AF-2 and thereafter conformational changes create a proper surface for interaction with transcriptional factors and putative mediators (Danielian et al. 1992, McInerney et al. 1996).

The DNA binding domain contains two zinc finger motifs. Each contains four cysteine molecules. This results in a tertiary structure which contains helices that interact with DNA sequences. The DNA binding domain can be divided into two subdomains. The helix of the first subdomain is involved in site-specific recognition. The P box is part of the first subdomain and is responsible for site-specific discrimination of binding. The second subdomain has been shown to act as a homodimerization interface and can act by unspecific DNA interactions (Luisi et al. 1991, Lee et al.

1993). The third helix of the RXR (retinoid X receptor) DBD after the second zinc finger has been suggested to function in homodimerization and interact with other members of the superfamily (Rastinejad et al. 1995).

The structure of the N terminal domain is not well known. It contains a transactivation domain, activation function AF-1, that plays an important role in gene regulation and hydrophobic residues of AF-1 are important in transcriptional activation. It contains ligand independent activation function. How it interacts with other proteins to induce transcription is not known (Giguere et al. 1986, Hollenberg et al. 1987, Dahlman-Wright et al. 1995).

The nuclear localization of the nuclear receptor is mediated by different signal sequences. One is in the hinge region (D domain). The D domain contains part of the sequence forming constitutive active NLS and C and E domains are also involved in the nuclear localization of the receptor. The NLS of PR have been precisely mapped and it has been suggested that with the ligand they play a role in nuclear import and export (Ylikomi et al. 1992, Beato and Klug 2000).
All the nuclear receptors which have been studied so far are considered to be phosphoproteins. It has been suggested that phosphorylation plays a role in receptor activation (Dougherty et al. 1982, Housley and Pratt 1983, Grandics et al. 1984, Wu et al. 2005).

The three-dimensional structures of NRs are very similar. X-ray crystallography has shown that LBD has a structure of 12-α helices (H1-H12) and one β turn that are arranged into three anti-parallel layers with a pocket into which the ligand fits. Ligand recognition is achieved through a combination of specific hydrogen bonds and the complementarity of the hydrophobic residues which line the cavity to the non-polar nature of ligands. Ligand-binding in the pocket alters the conformation of LBD with helix 12 forming a lid over the pocket, traps the ligand in the hydrophobic environment and forms a surface with which the coactivators interact. Helix 12 is important for AF-2 function, because it surrounds the core of AF-2. Crystal structures of NR LBDs have been described for example dimeric holo and antagonist bound ERα, ERβ, monomeric holo-RARgamma, heterodimeric complex of RAR, heterodimeric complex of RXR (Moras and Gronemeyer 1998, Gustafsson 2005).

1.2. Progesterone receptor

Progesterone is an essential regulator of the reproductive events associated with the establishment and maintenance of pregnancy, ovulation, uterine and mammary gland development, and neurobehavioral expression (McKenna et al. 1999). The effects of progesterone are mediated by specific intracellular proteins termed progesterone receptors. Progesterone receptor (PR) was purified from chicken oviduct cytosol by chromatography and by this method the first monoclonal chicken progesterone receptor antibodies were prepared (Sullivan et al. 1986). In cells the progesterone receptor is found in two variants, A and B forms. Human and chicken have two isoforms (A, M=72000 and B, M=86000 in chicken and A, M=90000 and B, M=120000 in humans) (Jeltsch et al. 1990). The PR-A and –B isoforms differ in that PR-B contains additional amino acids in its amino terminal half. The length of this region is 165 amino acids in mouse but varies from 128 to 165 amino acids depending on the species. This region encodes the transactivation function that is specific to the PR-B (Horwitz 1992, Giangrande and McDonnell 1999). Both A and B receptors can bind progesterone, dimerize, interact with hormone-responsive element and affect transcription. A and B proteins can dimerize as three (A-A and B-B homodimers and A-B heterodimers) species (Conneely and Lydon 2000). Functionally these isoforms differ (Tora et al. 1988, Kastner et al. 1990, Meyer et al. 1992, Vegeto et al. 1993, Giangrande and McDonnell 1999, Conneely et al. 2003). There are reports where PR-B functions as an activator in the transcription of several PR-dependent promoters and in different cell types when PR-A is inactive. When A and B

proteins are coexpressed in cell culture where agonist bound PR-A was inactive, the PR-A form could act as repressor of PR-B-mediated transcription (Vegeto et al. 1993, Kraus et al. 1995). The repressive function extends to other steroid receptors (estrogen, glucocorticoid and mineralocorticoid) through competing for limiting coactivators (McDonnell et al. 1994, Wen et al. 1994). Antagonist-bound PR-A is inactive but antagonist-bound PR-B can modulate intracellular phosphorylation pathways in PR-B cells (McDonnell and Goldman 1994, Wen et al. 1994). When both are antagonist bound they can repress ER activity but this is more specific to PR-B form (Kraus et al. 1995).
The phosphorylation of chicken progesterone receptor (cPR) has been most thoroughly studied. Four phosphrylation sites have been characterized in cPR and they contain Ser-Pro motifs. Two of their sites Ser211 and Ser260 are phosphorylated in the absence of hormone, but phosphorylation is increased 2-fold in the presence of progesterone. The other two, Ser530 and Ser367, are phosphorylated in the presence of hormone. Ser530 is in the hinge region. This region is occluded in the receptor-Hsp90 complex. Mutation in one of the ligand dependent motifs decreases transcriptional activation at low concentrations of ligand but at higher concentrations this does not have any effect on receptor activity. In the absence of ligand cPR can be activated by tyrosine phosphorylation and dopamine receptors. Human PR does not exhibit ligand independent activation. There is evidence that it has dozens of phosphorylation sites. It may be phosphorylated in the absence of ligand, but ligand increases it. The final phosphorylation of Ser-Pro sites in hPR requires DNA binding. Human PR contains phosphorylation sites at serine 81, 162, 190 and 400, which are representing basal sites and hormone-induced sites are serine 102, 294 and 345. Human PR contains various serine phosphorylation sites that are potential substrates for kinase signaling pathways (Weigel et al. 1995, Lange 2004, Narayanan et al. 2005).

1.3. Estrogen receptor

Estrogens affect growth, development, and differentation, including important regulation of the reproductive systems in male and female, mammary gland development and differentiation and act as anti-atherosclerotic agents, and act in the central nervous system functions and in the regulation of hypothalamic-gonadal axis. Estrogens mediate these effects through two intranuclear receptors estrogen receptors (ER) ERα and ERβ. They have some structural and functional similarities in common with other members of the steroid receptor superfamily. In DNA binding domain Estrogen receptors α and β have 96% amino acid identity but in the ligand binding domain homology is 53%. In the N-terminal AF-1 and C-terminal AF-2 there is less conservation between these two receptors. This suggests that different proteins may interact with ERα and ERβ during transcription and explains why these receptors function oppositely (Paech et al. 1997). Uterus, mammary gland, placenta, liver, bone, central nervous system and cardiovascular system tissues have high ERα content whereas ERβ is highly expressed in prostate epithelium, urogenital tract, lung, ovarian follicles, intestinal epithelium (Gustafsson 1999) (Matthews and Gustafsson 2003, Koehler et al. 2005). ER sediments at 8S when cytosol is extracted with low salt buffer and at 4S with high salt buffer. ERβ sediments at 4S regardless of the salt concentration is used in the buffer (Tate et al. 1983, Saji et al. 2000, Weihua et al. 2001, Palmieri et al. 2002). There are indications that in low salt buffer ERβ does not interact with those chaperones which normally interact with ERα (Weihua et al. 2003). Estrogen response element is directly bound by ERα and ERβ but ERβ is a weaker transactivator (Mosselman et al. 1996, Cowley et al. 1997, Pettersson et al. 1997, Watanabe et al. 1997, Ogawa et al. 1998). Crystallographic studies have shown that the ligand binding domains of ERα and ERβ have a similar overall architecture. In the absence of hormone ER is an inactive, repressed complex by molecular chaperones whereas ligand binding changes the position of helix12 and releases receptor from the inactive complex. This facilitates homodimerisation and binding to specific DNA sequences in the regulatory regions. The exact mechanism by which ER affects gene

transcription is poorly understood. When ERα and ERβ are coexpressed ERβ inhibits action on ERα-mediated gene expression (Matthews and Gustafsson 2003).

1.4. Interaction of PR and ER

Migliaccio A et al. 1996 were first to report interaction between PR, ER and p60-Src kinase in T47D cells (Migliaccio et al. 1996). Ballare C et al. 2003 reported that mitogen-activated protein kinase (MAPK) activation by progestins is blocked by antiprogestins and antiestrogens in cells transfected with both PR and ER. It was suggested that c-Src/MAPK activation by PR is mediated indirectly by the interaction of the Src-homology domain of c-Src with phosphotyrosine 537 of ERα. The activation of c-Src and MAPK pathway by progestins is dependent on the presence of unliganded ERα, which interacts with PR-B via two domains that flank PR`s proline rich sequence. The deletion of either of these PR-B domains, which interacts with ER blocked c-Src/MAPK activation by progestins in the presence of ERα (Ballare et al. 2003). However there is report, which has shown that PR expression increased c-Src activity in Cos-7 cells in the absence of progestins and independently of added ER. Coexpression of PR and ERα reduced basal levels of c-Src activity (Boonyaratanakornkit et al. 2001). The reasons for the discrepancies are unknown.

2. RECEPTOR ASSOCIATED PROTEINS

2.1. Chaperone complex

Steroid receptors form large oligomeric complexes in the absence of hormone and under hypotonic conditions (Toft and Gorski 1966, Gorski et al. 1968, Grody et al. 1982, Smith et al. 1990). Most of the receptor associated proteins belong to chaperoning proteins. In eukaryotes molecular chaperones are essential proteins that take part in the regulation of steroid receptors. Chaperones bind and stabilize unstable forms of protein and facilitate the folding of the protein, oligomeric assembly, transport in the cell compartment, switch between active and inactive conformations and assist in the regulation of signal transduction pathways. Their function is to ensure that the assembly of other polypeptide chains occurs correctly. They do not determine the tertiary structure of the folding protein and prevent incorrect interactions of protein folding intermediates. Chaperone-bound receptor is stabilized so that it has high affinity for hormone but cannot bind DNA. Many molecular chaperones are called heat shock proteins (Hsps) (Hartl 1996). See Table 1 (Johnson and Toft 1994, Chen et al. 1996, Dittmar et al. 1997, Dittmar et al. 1998, Morishima et al. 2000, Morishima et al. 2001, Pratt and Toft 2003).

Protein	Function	Reference
Hop	• binds to Hsp70 and Hsp90 and brings them together •increases steroid-binding activity	Chen S. et. al. 1996 Morishima Y. et. al. 2000
Hsp40	• binds to Hsp70 and directly to the receptor •increases steroid-binding activity	Dittmar KD et. al. 1998
Hsp70	• essential for receptor-Hsp90 assembly •opens with Hsp90 steroid-binding cleft	Morishima Y et. al. 2001
Hsp90	• binds to the receptor and Hop •regulates steroid receptor with multiprotein complex	Pratt WB and Toft DO 2003
Immunophilins	• role unclear •binds to Hsp90 and essential for Hsp90 function	Johnson JL et. al 1994
p23	•binds to Hsp90 •stabilizes receptor-Hsp90 complex	Dittmar KD et. al. 1997

Table 1. The functions of the proteins in the oligomeric complex.

2.1.1. Hsp40/DnaJ/Ydj1

Hsp40 is a co-chaperone. It binds to the ATPase domain of Hsp70 and can stimulate ATP hydrolysis activity. Hsp40 is involved in early steroid receptor heterocomplex formation. Hsp40 (Ydj1) has been shown in yeast studies to be essential for Hsp90 activity in vivo and interaction between Ydj1 and Hsp70 is critical for the maturation of Hsp90 substrates. Ydj1 is a homolog of bacterial DnaJ because it contains all the functional domains of DnaJ (Johnson and Craig 2000). There is clear evidence that Hsp40 binds directly to PR in the absence of Hsp70 and binds to PR in a static manner during steroid cleft opening (Hernandez et al. 2002). Stoichiometry of Hsp40 is 1:1 in the complex. Hsp40 carries out in the complex ATP, Mg^{2+} and K^{+}-dependent opening of the steroid binding cleft. Addition of Hsp40 and Hop increases steroid binding activity (Pratt and Toft 2003).

2.1.2. Hsp90

Hsp90 is an abundant cytosolic protein. Almost 100 proteins are known to be regulated by Hsp90 but it is not known how they are regulated. It has been suggested that in vitro in the absence of hormone nuclear receptors form a heterocomplex with Hsp90 and in the presence of hormone the complex dissociates. Hsp90 forms a direct contact with the receptor. Hsp90 acts on the ligand-binding domain of the steroid receptors. The multiprotein complex with Hsp90 regulates steroid receptor function. Hsp90 has a single TPR (tetratricopeptide repeat) recognition site, which is why immunophilins and Hop compete for binding to Hsp90. p23 binds to a separate site on Hsp90. The location and number of binding sites on Hsp90 are still uncertain. On its own Hsp90 is not capable of binding steroid receptors and needs Hsp70 and Hop (Pratt et al. 2004a).

2.1.3. Hop/Sti1

Hop (Hsp-organizing protein) is a 60-kDa protein and is a mammalian ortholog of yeast protein Sti1 (Nicolet and Craig 1989). It has been suggested that Hop is transiently a part of the complex during assembly, but it is not a component of functionally mature PR complexes. It can bind to Hsp90 and Hsp70 independently and in an ATP-independent manner and it is a central factor of the Hsp90-Hop-Hsp70 complex. N-terminal TPR (tetratricopeptide repeat) domain is required for Hsp70-binding and central TPR domain for Hsp90 binding. Hop binds to C-terminal of Hsp90, TPR acceptor site. Hop physically links the activities of Hsp90 and Hsp70 and also modulates the activities of these two chaperones. The mechanism by which Hop affects Hsp70 function is unknown. Hop blocks ATP binding and the ATPase function of Hsp90 and also inhibits binding of p23 to Hsp90. In reticulocyte lysate there are few complexes which contain Hop. Steroid binding for PR and GR decreased 60% when Hop was eliminated (Chen and Smith 1998, Pratt and Toft 2003).

2.1.4. Hsp70

Hsp70 is essential for the assembly of signaling protein-Hsp90 heterocomplex. Hsp90 and Hsp70 interact directly when opening steroid binding-cleft in the GR. Mammalian Hsp90 and Hsp70 cannot form stable complexes to be detected by biochemical separation techniques. The interaction has been shown by crosslinking studies. Where Hsp70 binds to Hsp90 remains unknown. When Hsp70 or Hsp90 is absent from the complex no steroid-binding activity is generated. It has been shown that the binding sites for Hsp90 and Hsp70 could not be separated. Some Hsp70 is released during complex assembly. The stoichiometry of Hsp70 in the final complex is less than 1:1 to the receptor. Hsp70 binds to peptides that are unfolded. Hsp70 can not be dissociated from receptors by hormone or salt. It is associated with transformed receptor when it binds to DNA, but does not affect DNA binding activity (Pratt and Toft 1997).

2.1.5. p23

p23 is acidic 23 kDa protein. It binds to ATP-dependent conformation of Hsp90. Its binding domain on Hsp90 is unknown. p23 stabilizes receptos-Hsp90 heterocomplex. The binding to Hsp90 takes place late in the chaperoning pathway, when Hop has dissociated from the complex and Hsp90 has assumed ATP-bound conformation. The binding requires elevated temperature, presence of ATP and molybdate (stabilizes Hsp90). p23 and Hsp90 interaction is dependent on the conformation. Mutations of Hsp90 effects that p23 loses binding activity. The function of p23 is unclear but it can inhibit aggregation of denatured proteins (Johnson and Toft 1995, Morishima et al. 2003, Pratt and Toft 2003).

2.1.6. Hip/p48

Hip is a 48-kDa protein recovered in the reticulocyte lysate of PR heterocomplex. It is cochaperone of Hsp70. It binds to ATPase domain of Hsp70. In some studies Hip is considered an important functional component but other studies have indicated that it is not essential for the assembly of the heterocomplex (Smith 1993, Pratt and Toft 2003).

2.1.7. Immunophilins

Immunophilins bind immunosuppressant drugs (FK506, rapamycin, cyclosporin A) and they can be divided into two classes: 1) the FKBPs (FKBP51, FKBP52) are binding proteins for FK506 and 2) rapamycin and cyclophilins (Cyps) bind cyclosporin A. FKBP52, FKBP51 and CyP40 have been shown to be components of the steroid receptor-Hsp90 heterocomplex. Larger immunophilins have several TPR (tetratricopeptide repeat) domains and calmodulin-binding domain in the C-terminal region. Hsp 90 binds via TPR to immunophilins. Hop and TPR domain immunophilins compete with each other for binding to TPR acceptor site located at C-terminus of Hsp90. CyP4, FKBP51 and FKBP52 bind 2 molecules of TPR protein to Hsp90 dimer. Immunophilins are not required for the generation of an active steroid receptor or for stabilization of the receptor. It has been suggested that immunophilin binding to Hsp90 is essential for the action of immunophilins which have TPR domain and for the function of Hsp90 (Pratt and Toft 1997, Galigniana et al. 2004, Pratt et al. 2004a). Immunophilins have in common peptidyprolyl isomerase domain (PPIase). The PPIase domain of the Hsp90-binding immunophilin links steroid receptors to the cytoplasmic-nuclear trafficking system. Immunophilin links the Hsp90-heterocomplex via PPIase domain to cytoplasmic dynein for retrograde movement along the microtubules towards the nucleus. Dynein is a 1.2 MDa multisubunit complex (Zydowsky et al. 1992).

2.2. Cofactors

2.2.1.Coactivators and corepressors

Coactivators and corepressors enhance or repress the activity of genes by nuclear receptors in a ligand-dependent manner. Coactivators interact with activation domain of the nuclear receptor directly in an agonist-dependent manner and this leads to the enhancement of the receptor activation function. The Coactivator also interacts with components of the basal transcription machinery but it does not enhance transcription activity on its own (Robyr et al. 2000). Coactivators can be divided into two classes: 1) members of the switch/sucrose nonfermentable (SWI/SNF) family and 2) members of the histone acetyltransferase family. These 2 classes can modify chromatin (Collingwood et al. 1999). SWI/SNF proteins were characterized as regulators of genes in yeast

(Neigeborn and Carlson 1984). Transcription activation of GR in yeast was facilitated by SWI/SNF (Yoshinaga et al. 1992). SRC-1 (steroid receptor co-activator 1) was the first protein to interact with PR in a ligand-dependent fashion (Onate et al. 1995). It has several variants (SRC-1b,-c,-d and –e). SRC-1e is a more potent activator for ER than SRC-1a (Kalkhoven et al. 1998) but they both have 3 nuclear receptor-interacting motifs which are found in many co-factors (Heery et al. 1997). The difference in activity results from the two distinct activation domains of SRC-1. The way in which the transcription activation signal is transmitted to the transcriptional machinery is unknown (Robyr et al. 2000). Coactivators CBP (CREB-binding protein) = cyclic AMP response element/p300 also bind to the nuclear receptor in a ligand-dependent manner and enhance transcription with SRC-1 in synergy (Smith et al. 1996). p/CIP (p300/CBP cointergrator-associated protein) and P/CAF (p300/CBP associated factor) are co-activators which can associate to CBP/p300 (Yang et al. 1996, Torchia et al. 1997, Blanco et al. 1998). In many cell lines p300/CBP, p/CIP and SRC-1 together allow ligand-activated gene transcription (Torchia et al. 1997). P/CAF, p300/CBP and SRC-1 have histone acetyltransferase activity (HAT) which correlates with promoter activation (Rubin et al. 1996). Histone acetylation facilitates transcription factor access to DNA and transcription. Some coactivators share sequence homolog. SRC-1 is homologous to TIF2 (transcription intermediary factor 2) and RAC3 (receptor associated coactivator 3). ARA70 (androgen receptor-asscociated protein 70) enhances AR activity more than other steroid receptors. Coactivators are a growing family of proteins (Jenster 1998).

Transcriptional repression requires several mechanisms (Johnson 1995). A Repressor can bind directly to DNA and prevent coactivator binding or can directly silence the basal transcription machinery. Repression occurs in the absence of hormone or the antagonist is bound to a receptor. The corepressor interferes directly with the transcription machinery (Robyr et al. 2000). There are 2 classes of corepressors: 1) N-CoR (nuclear hormone receptor corepressor and SMRT (silencing mediator of retinoid and thyroid receptors) and 2) TIF1 (transcription intermediary factor-1). Both classes function to recruit histone deacetylase close to the receptor. N-CoR and SMRT interact with unliganded TR and RAR associated with the RXRheterodimeric partner on DNA (Collingwood et al. 1999, Robyr et al. 2000). N-CoR and SMRT repress transcription, at least in part, by binding to histone de-acetylases (HDACs) either directly or indirectly through other corepressor complex components (Webb et al. 2003). Both N-CoR and SMRT interact with the nuclear receptors through the RIDs (receptor-interacting domain) located in the C-terminal of the proteins, while their transcriptional repression domains are in the N-termini. N-CoR/SMRT also associates with HDAC3 (histone deacetylase 3) in large protein complexes, which is an important pathway for transcriptional repression. Corepressors N-CoR and SMRT interact with the NRs either in the absence of agonists (TR and RAR), or in the presence of antagonists (steroid receptors) (Li et al. 1997, Gao et al. 2002, Kumar et al. 2005).

3. HEAT SHOCK PROTEINS

Increased expression of heat shock proteins occurs when cells are exposed to increased temperature, metabolic insults, heavy metals, ionophores and other poisons. Heat shock proteins are essential for the survival of the cell when the cell encounters environmental insult. In mammalian cells heat shock proteins exhibit masses of 8, 28, 58, 72, 73, 90 and 110kDa (Welch 1992). The major families are small stress proteins, Hsp40, Hsp60, Hsp70, Hsp90 and Hsp110 families. Most of the stress proteins are expressed in cells under normal growth conditions. It has been shown that heat shock proteins act as chaperones and facilitate protein folding. They also act as markers for cell damage, are related to immune response and are considered a tool for toxicology (Garrido et al. 2001, Soti et al. 2005).

3.1. Hsp110

Hsp110 is the third or fourth most abundant Hsp in most mammalian cell lines and tissues and is sometimes called Hsp105. Hsp110 accounts for 0.7% of total cell protein after stress reaction. Its expression is high in liver and brain but low in heart and muscle. It prevents protein aggregation of denatured proteins in vitro. It is localized in the nucleus and in the cytoplasm and has been found in conjunction with Hsp70 in the cytoplasm and nucleus. Hsp110 does not bind ATP in vitro. The role of Hsp110 in cellular physiology is little known (Easton et al. 2000).

3.2. Hsp70

At least Hsp72, Hsp73, Hsp75 and Hsp78 belong to the Hsp70 group. They have common protein sequences but are synthesized in response to different stimuli (Kregel 2002). The Hsp70 family has been considered to be one of the most conserved gene families in biology (Boorstein et al. 1994), because members have approximately the same size and have limited sequence divergence. The Hsp70 family is the most temperature sensitive. It binds ATP. Several functions have been attributed especially to Hsp70. Hsp70 acts as chaperone, maintains the structure of proteins, refolds misfolded proteins, translocates proteins across membranes, prevents aggregation of proteins and degradates unstable proteins. Hsp73 is constantly produced whereas Hsp72 is highly inducible and its synthesis is increased in response to multiple stressors. Hsp70 has been associated with the development of tolerance to variety of stresses; hypoxia, ischemia, acidosis, energy depletion, ultraviolet radiation (Pratt and Toft 1997, Bukau and Horwich 1998, Kregel 2002). In unstressed cells Hsp70 is diffusely located in the cytoplasm and the nucleus, but in the presence of heat shock there is an increase of protein in cytoplasm and nucleus and concentration in the nucleoli (Welch and Feramisco 1984). Hsp70 may inhibit apoptosis (Garrido et al. 2001).

3.3. Hsp60

Mammalian Hsp60 (chaperonin) is mostly located in the mitochondrial matrix. It participates in the folding of mitochondrial proteins and degradation of misfolded proteins or denatured proteins. It is dependent on ATP. Hsp60 is regulated by Hsp10. It also has a proapoptotic role during apoptosis (Garrido et al. 2001).

3.4. Hsp40

Ydj1 is a yeast homolog in the Hsp40 family. It contains all the functional domains of bacterial DnaJ (Johnson and Craig 2000). It is also homologous to HDJ2, which is human Hsp40 protein (Hernandez et al. 2002). It has been shown that Hsp40 is the first protein that binds to PR and remains bound to PR throughout the chaperoning system. It is not dependent on ATP (Pratt and Toft 2003). Hsp40 binds to Hsp70 and stimulates its ATP hydrolysis activity (Cyr et al. 1994).

3.5. Small Hsps

Small Hsps vary in size from 15 to 30kDa (Hsp32, Hsp27, αβ-crystallin and Hsp20) and share sequence homologies and biochemical properties such as phosphorylation and oligomerization. Hsp27 is an ATP-independent chaperone. Its main function is to protect against protein aggregation. (Garrido C.et al 2001) Hsp27 has antiapoptotic activity (Jakob and Buchner 1994, Garrido et al. 2001).

3.6. Hsp90

Heat shock protein 90 (Hsp90) is one of the most abudant proteins in mammalian cells, accounting for 1-2% of cytosolic protein. Its synthesis increases after stress. In response to stress expression of

Hsp90 increases up to tenfold in prokaryotes and eukaryotes. Hsp90 is present in the cytoplasm of eubacteria, yeast and mammals (Buchner 1999). In yeast high concentrations of Hsp90 are required for growth at high temperatures and Hsp90 is essential at all temperatures in yeast. In E. coli Hsp90 is not essential not even under stress (Bardwell and Craig 1988, Borkovich et al. 1989).Two cytosolic isoforms of Hsp90 are called Hsp90α and Hsp90β. They are expressed differentially (Moore et al. 1989). Human Hsp90α has 86% amino acid homology with Hsp90β (Hickey et al. 1989). Chicken Hsp90 has 64-96% sequence homology from yeast to man (Binart et al. 1989).

It has been shown that Hsp90 has a modular structure in which two well-conserved regions are connected by a linker of variable length. The linker is not essential for Hsp90 function. The protein is an elongated dimmer. The association sites lie in the C-terminal region of the protein. The Dimer seems to be flexible and different shapes of the molecule can be detected. The Dimerization domain is at the C-terminus (Palmer et al. 1995, Maruya et al. 1999). N-terminal domain has been crystallized and has been found to contain a binding site for nucleotide and geldanamycin. Geldanamycin has been shown to inhibit Hsp90 action. N-terminal domain is an α-β sandwich with a ligand-binding pocket. There are 8β sheets and 9α helices and intervening loops (Prodromou et al. 1997). Heat shock temperatures have been shown to trigger an additional and specific structural transformation in Hsp90. Then the N-terminal domains come into close proximity (Buchner 1999).

Hsp90 has been shown to bind steroid receptors and dioxin receptor. It stabilizes the high-affinity ligand-binding form, binds pp60, inhibits tyrosine kinase activity, binds ATP and autophosphorylates, binds casein kinase II and prevents its aggregation. The list of proteins whose folding is affected by Hsp90 in vivo has been growing in recent years. Many of these are transcription factors, kinases and several are not related to these classic clients (Pratt and Toft 2003).

Hsp90 has an ability to stimulate folding by other chaperones. It can bind to peptides by itself and is capable of stabilizing unfolded proteins and protecting them from aggregation. In a limited capacity it functions in protein-folding reactions. Thus Hsp90 can function as a molecular chaperone. It also has a role in the activation and assembly of a range of client proteins involved in signal transduction, cell cycle control or transcriptional regulation (Pratt 1998). Hsp90 interacts with co-chaperones, whose number is increasing, but whose role is unknown. It has been suggested that these processes occur through conformational changes in Hsp90 and hydrolysis. Hsp90 has been shown to have an essential role in ATP binding and hydrolysis in vivo (Obermann et al. 1998). Hsp90 binds to short hydrophobic peptides and can maintain protein in folded state (Buchner 1999, Holt et al. 1999). The primary binding site for TPR (tetratricopeptide repeat) has been shown to localize in the C terminus of Hsp90 (Young et al. 1998). Co-chaperones of Hsp90 have been shown to contain multiple copies of TPR (Chen et al. 1998). There is evidence that Hsp90 dimer can bind two TPR-containing co-chaperones and there are two binding sites for TPR in Hsp90 dimer (Carrello et al. 1999, Russell et al. 1999, Smith 2004).

Hsp90`s association is required for the stability and function of multiple signaling proteins that promote the growth and/or survival of cancer cells. The number of these proteins has grown dramatically during the past years and includes v-Src tyrosine kinase, Bcr-Abl kinase, Raf-1 kinase, Akt kinase, ErbB2 transmembrane kinase, mutated p53 and transcription factors including steroid receptors and hypoxia-inducible factor 1α (HIF-1α) (Neckers 2002). See Figure 2. It has been shown that Hsp90 can bind to Akt, which has been shown to phosphorylate IκB kinase and upregulates its kinase activity, which results in promotion of NF-κB mediated inhibition of cell death (Sato et al. 2000). Raf-1 mediates signalling by stimulation of mitogen-activated protein kinase pathway (Schulte et al. 1995). Geldanamycin and radicicol are Hsp90 inhibitors which cause

destabilization and degradation of Hsp90 client proteins. Geldanamycin replaces nucleotide in the Hsp90-binding pocket with affinity greater than ATP or ADP and directs assembly of chaperone machine, which causes client protein degradation. Hsp90 inhibitors have shown promising antitumor activity in preclinical model systems (Neckers 2002, Chiosis et al. 2004).

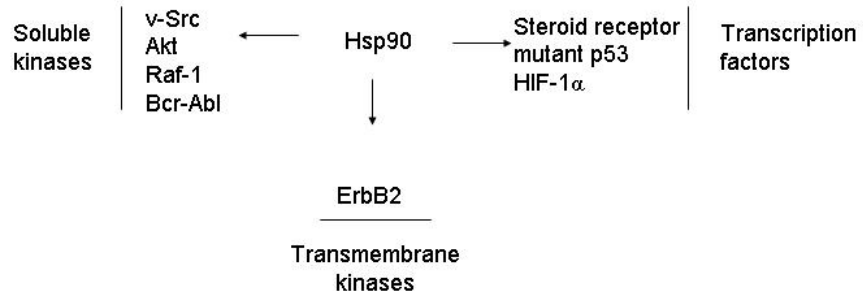


Figure 2. Hsp90 interferes with multiple signaling pathways. Soluble kinases are v-Src, Akt, Raf-1 and Bcr-Abl. The transmembrane kinase is ErbB2. Transcription factors include steroid receptors, mutant p53 and HIF-1α

4. REGULATION OF NUCLEAR-CYTOPLASMIC LOCALIZATION OF PROTEINS

4.1. Nuclear localization

4.1.1. Nuclear import

Nuclear proteins enter the nucleus after synthesis in the cytoplasm. The nucleus is surrounded by a double membrane. The transport of molecules across the nuclear membrane occurs through nuclear pore complexes (Dingwall and Laskey 1992). About 100 proteins constitute a nuclear core complex (Forbes 1992, Stewart 1992). The Proteins of the nuclear pore complex are called nucleoporins. Several nucleophorins have been described (Nsp1p, Nup49p, Nup57p, Nic96p) (Stochaj and Silver 1992, Yamasaki and Lanford 1992). Molecules enter the nucleus by two mechanisms: passive duffusion of small molecules (40-60kDa) (Peters 1986) and active transport of large molecules. Larger molecules require ATP to enter the nucleus (Richardson et al. 1988). Electron microscope studies have shown that the pore complex has a diameter of 80-100nm and 125MDa molecular mass (Unwin and Milligan 1982, Akey 1989, Reichelt et al. 1990). Nuclear localization signals (NLSs) are responsible for targeting proteins into the nucleus (Chelsky et al. 1989, Gorlich and Mattaj 1996, Nigg 1997). The first characterized NLSs were single SV40 large T antigen NLS (Lanford et al. 1986) and bipartite nucleoplasmin NLS (Robbins et al. 1991). In vitro assay for nuclear transport using digitonin-permeabilized cells has yielded an advance in nuclear import, NLS studies (Adam et al. 1990). The Proteins carrying NLS are called importin-α/β (also karyopherin-α/β, pore targeting complex) and transportin. There is low sequence identity between transportin1 and importin-β but the overall structure is similar. Importin-α (55-kDa protein) binds to NLS-proteins and importin-β strengthens the affinity of the complex for NLS (Nigg 1997). The transfer of the protein-carrier complex through NPC requires a cytoplasmic Ran-GDP and Ran-GTP/GDP cycle. Ran is the most abundant member of the Ras family. This translocation step is little known

(Melchior et al. 1993, Moore and Blobel 1993, Pemberton and Paschal 2005). When the complex has reached the nucleoplasmic side of the NPC, NLS protein is released. Importin-β is returned to the cytoplasm and importin-α and NLS-protein are released into the nucleus (Nigg 1997). In ER one estrogen-inducible proto-NLS has been found in the hormone binding domain and three constitutive NLSs have been characterized. In PR three proto-NLS and one weak inducible proto-NLS have been found (Ylikomi et al. 1992).

4.1.2. Nuclear export

Classic studies of nuclear export have focused on the different species of RNA moving out of the nucleus. Protein export from the nucleus is a specific signal-directed event (Corbett and Silver 1997). Nuclear export has been studied with large Balbiani ring transcripts in the salivary glands of the insect Chironomus tentans representing an object for morphological studies and nuclear export of messenger RNPs (ribonucleo protein particles). Microinjection of proteins or RNAs into the nucleus of Xenopus oocytes has shown that RNA export is enery-dependent. Analysis of yeast mutant strains has revealed defects in RNA export. Viral-Rev protein is encoded by HIV-1. Rev directs the nuclear export of RRE(Rev response element)-containing RNAs. Within C-terminal domain Rev contains nuclear export signal (NES) (Nigg 1997). Crm1 (exportin1, Xpo1) was the first export carrier identified. It recognizes a short motif rich in leucine or related hydrophobic residues, which is found in Rev. Isolates of Crm1 have low affinity for Ran-GTP and most NES cargoes. Crm1-cargo complex exits the nucleus through NPCs and is dissociated by hydrolysis of Ran-GTP. Cytoplasmic calreticulin is an export carrier for steroid receptors. It can bind directly GR and it is independent of Crm1 (Macara 2001). In PR-B 2 putative NESs have been found, one present in the N-terminal of PR-B and absent in PR-A, the other was found in the region common to A and B forms (Kanwal et al. 2002).

4.1.3. Shuttling

Steroid receptors have nuclear localization signals whose cellular localization and transcriptional activating activity depend on hormonal regulation. They shuttle constantly, passing into and out of the nuclei in the absence of hormone. The localization of steroid receptors is not static (Guiochon-Mantel et al. 1996). Hormone-free receptors are associated with Hsp90. It has been suggested that traffic through the cytoplasm into the nucleus is possible in association with Hsp90 (Pratt 1990). Chicken Hsp90 was imported into the nucleus by fusion with nucleoplasmin NLS. PR and GR mutants which lack NLS localized in the cytoplasm. When steroid receptor mutants lacking NLS were co-expressed with Hsp90-NLS, there was complete nuclear localization of the receptors (Kang et al. 1994). Molybdate has been shown to stabilize GR-Hsp90 complexes in vivo. It also inhibits the hormone-dependent nuclear import of GR. In the presence of molybdate receptors can be exported from the nucleus but cannot be re-imported into the nucleus (Yang and DeFranco 1996). Re-association of receptors with Hsp90 in the cytoplasm is needed so that the receptor can recycle to the nucleus (Pratt and Toft 1997).

4.2. Cytoplasmic localization

Nucleocytoplasmic signaling of proteins is based on anchoring and release and masking and unmasking of NLSs. These two models are activated by ligand binding, phophorylation or proteolysis. Membranes are involved in the cytoplasmic anchoring. Inactive PKA (cAMP-dependent protein kinase) is anchored to cytoplasmic membraneous organelles such as Golgi complex, microtubules, plasma membrane, mitochondria (Nigg 1997, Martin et al. 1999). MAGUKs (membrane-associated gyanylate kinase homologs) have been found to be involved in subcellular targeting. They consist of protein domains: PDZ1-3 (Dlg), SH3, HOOK, and GUK.

HOOK is required for the localization of protein in the cell membrane and PDZ2 is needed to restrict the protein to the separate junction. HOOK is a targeting signal for Dlg. In the presence of HOOK Dlg localizes to the membrane (Hough et al. 1997). Maternal morphogen dorsal (dl) is localized in the cytoplasm. C-terminal of dl is important in the retention of dl in the cytoplasm (Rushlow et al. 1989). Rel and NF-κB are transcription factors, which are present in most mammalian and avian cells. In unstimulated cells the homo- or heterodimeric Rel/NF-κB are cytoplasmic proteins by binding to inhibitor protein (IκB), which somehow masks the NLSs (Latimer et al. 1998).

4.3. Subcellular localization of nuclear receptors

The cellular activities of steroid hormones are mediated by nuclear receptors. The first studies using cell fractionation technique proposed that unliganded steroid receptors are located in the cytoplasm and translocate into the nucleus after binding with the hormone (Gorski et al. 1968, Jensen et al. 1968). Various histochemical techniques have been applied to discover the localization of nuclear receptor in specific celltype. Immunohistochemical studies have concluded that steroid receptors are located in the nucleus independent of the hormonal status (King and Greene 1984). ERα has been shown to localize in the nucleus in the female reproductive organs and in cell transfected with ERα. ERβ has also been shown to localize in the nucleus independent of hormonal status by immunocytochemistry (Hiroi et al. 1999). Chick PR-B has been shown in transfected HeLa cells to locate mainly in the nucleus (Ylikomi et al. 1992). Green fluorescent protein (GFP)-PRA was found in the nucleus but GFP-PRB was shown to localize in the nucleus and in the cytoplasm (Kawata et al. 2001). In chicken oviducts both PR isoforms have been shown to localize in the nucleus independent of hormonal status (Syvala et al. 1996). The subcellular localization of AR is controversial. GFP-AR was shown to localize in the cytoplasm in the absence of hormone and was partially translocated into the nucleus in the presence of hormone (Georget et al. 1997). Nuclear localization has been exclusively shown in AR in rat prostate, seminal vesicle and epididymis (Zhuang et al. 1992). The localization of GR is also controversial. GR has been shown to localize in the nucleus exclusively in the absence of ligand (Pekki et al. 1992). There are also studies showing that GR translocates from cytoplasm into the nucleus in the presence of hormone (Wikstrom et al. 1987). Exclusively nuclear localization vitamin D receptor has been shown by fractionation studies in the absence of hormone (Walters et al. 1981). Independent of hormonal status thyroid hormone receptor (TR) has been shown to localize in the nucleus in cell fractionation (Oppenheimer et al. 1976).

The entry of nuclear receptors into the nucleus requires NLSs. The localization of steroid receptors is not static, because receptors are constantly shuttling between nucleus and cytoplasm (Perrot-Applanat et al. 1992).

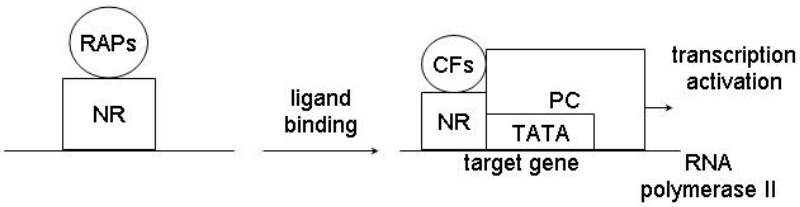
4.4. Subcellular localization of Hsp90

Most immunohistochemical studies have shown that Hsp90 is a cytoplasmic protein. There are also studies that have shown that Hsp90 is located in the nucleus and cytoplasm (Gasc et al. 1984, Gasc et al. 1990, Perdew et al. 1993). Chimeric DNA of Hsp90 and PR were transfected into HeLa cells and were seen exclusively in the nucleus in the presence and absence of hormone (Tuohimaa et al. 1993). In the chicken oviduct epithelium Hsp90 was shown in the cytoplasm in the presence or absence of progesterone. PR was localized in the nucleus (Pekki et al. 1995). Transfection studies showed that Hsp90 localizes in the cytoplasm but a small portion of Hsp90 translocates into the nucleus after coexpression with ER in COS cells (Meng et al. 1996). When chicken Hsp90 was targeted with 90NLS and human GR receptor was transfected with 90NLS into Cos cells, mutant GR was shown to shift into the nucleus in the absence of hormone (Kang et al. 1994). When Hsp90

and PR were transfected into HeLa cells Hsp90 was detected in the cytoplasm and PR in the nucleus (Tuohimaa et al. 1993).

5. TRANSCRIPTION REGULATION BY STEROID HORMONES

The initiation of transcription is a complex event occurring through the interaction of multiple factors which co-operate at the target gene promoter. In the absence of ligand the receptor is in an inactive state transcriptionally and is associated with other proteins (Smith et al. 1992). The receptor undergoes conformational change when binding ligand and dissociates from heat shock proteins. This allows the receptor to dimerize and bind to hormone responsive elements within the regulatory regions of target genes (McDonnell 1995). Agonist-bound PR has been shown to activate transcription by directly interacting with general transcription machinery and by associating with coactivators (Ing et al. 1992, Smith et al. 1996, Voegel et al. 1996). Coactivators SWI/SNF and histone acetyltransferase coactivators can modify chromatin and can influence the activity of the basal transcriptional machinery directly. Histone acetylation facilitates transcription factor access to DNA and transcription by destabilizing chromatin. Chromatin is composed of repetitive structural element called nucleosome. Nucleosomes must be disrupted over the TATA box so that basal transcription machinery can function (Collingwood et al. 1999). See Figure 3.



- Figure 3. A model how nuclear receptors (NR) are able to initiate gene transcription. In the presence of ligand RAPs (steroid associated proteins) dissociate and then nuclear receptor communicates with preinitiation complex (PC) and cofactors (CFs). PC consists of transcription factors,TATA-box binding protein (TBP) and TBP-associated factors.

After binding to specific DNA sequences, estrogen response elements ER interact with basal transcription factors, co-activators and other proteins. Once the transcription initiation complex is complete, RNA polymerase II is recruited to the transcription start site and transcription begins. RNA polymerase II can initiate transcription after basal transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH have collected together on the core promoter. TFIID consists of TATA box binding protein (TBP) and TAFs (tightly associated factors). ERα and PR interact directly with TFIIB. ERα also interacts directly with TBP using both AF-1 and AF-2 as interaction surfaces. ERα has been shown to interact with hTAF30 and hTAF28. It has been suggested that ERα has an intrinsic ability to activate transcription in a ligand-independent manner. Corepressors recruit a complex of proteins having histone deacetylase activity that repress gene expression by maintaining chromatin in a more condensed state. This prevents access by transcription factors to the template and represses transcription (Klinge 2000) (O'Lone et al. 2004).

The transcriptional activity of NRs can also be modulated by two other mechanisms. 1) Cell membrane mediated signal transducing pathways, which are modulated by polypeptide growth factors and dopamine and subsequently activate protein kinases, are thought to regulate the activities of NRs and interactive transcription cofactors through phosphorylation. For example, treatment with dopamine or inhibitors of cellular phosphatase can enhance the ligand-dependent activation of PR, ER and VDR (O'Malley et al. 1995). EGF, TGFα and IGF-1 bind to tyrosine kinase-associated membrane receptors. They have been shown to induce epithelial proliferation and synthesis of estrogen-inducible proteins in mouse uterus (Murphy and Ghahary 1990, Nelson et al. 1991, Nelson et al. 1992). 2) Through a mutual interference with other transcription factors such as activator protein-1 (AP-1), nuclear factor κB and signal transducers and activators of transcription 5(Stat 5). Interaction is assumed to be a direct protein-protein interaction (Cerillo et al. 1998, Stoecklin et al. 1999).

6. ROLE OF HSP90 IN THE STEROID HORMONE ACTION

Steroid receptors are transcription factors. The best studied substrates of Hsp90 are steroid receptors. 90kDa heat shock protein has been found in complexes with GR, ER, PR and AR. Thyroid hormone, retinoic acid and vitamin D do not form stable Hsp90 complexes (Pratt and Toft 1997). At the beginning it was suggested that hormone causes dissociation of receptor-Hsp90 complexes and generates active receptor (Gorski et al. 1968, Jensen et al. 1968). With steroid receptors it was shown that receptor activation included the conversion of 8S form to the 4S form of receptor (Jensen 1991).

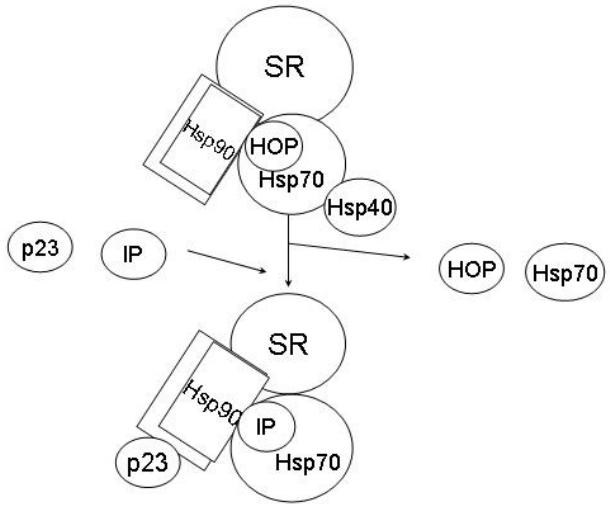
Many studies have mapped Hsp90 binding to the ligand-binding domain of steroid receptors (Gehring and Arndt 1985, Denis et al. 1988). The hormone binding domain of steroid receptors contains a nuclear localization signal that is repressed when the receptor is associated with Hsp90 (Kang et al. 1994). Free Hsp90 does not bind the receptor, but binding of Hsp90 to PR has been shown to be mediated by interaction with a pre-existing complex. Complexes between hsp90 and PR can be formed in vitro and in rabbit reticulocyte lysate. Thus dependent on ATP (Hutchison et al. 1992a, Stancato et al. 1993). Hsp90 association is required to maintain PR´s ability to bind progesterone at elevated temperatures. The ligand binding promotes conformational change and stability that influence receptor interaction with chaperones and association with transcription apparatus (Smith and Toft 1993).

Untransformed steroid receptor is unable to bind to DNA and to dimerize (Picard and Yamamoto 1987, Qi et al. 1989). There are three suggestions as to how Hsp90 inactivates steroid receptors: 1) Hsp90 maintains the receptor in an inactive state by blocking access to the DBD (Picard et al. 1988, Eilers et al. 1989) 2) binding of Hsp90 to the HBD causes the polypeptide to assume unfolded conformation (Picard et al. 1988, Yamamoto et al. 1988) 3) in the docking model hsp90 determines unfolded conformation of HBD. The dissociation of Hsp90 from steroid receptor leads to receptor transformation resulting in the unmasking of DBD, receptor dimerization sites and NLSs (Picard and Yamamoto 1987, Qi et al. 1989, Hsu et al. 1992).

In vitro studies have shown that Hsp90 is a chaperone and can chaperone the folding of proteins. Chaperone is a protein that binds to and stabilizes an otherwise unstable conformer of another protein and controls the binding and release of the substrate protein, facilitates its correct fate in vivo: folding, oligomer assembly and transport to a particular subcellular compartment or controls switch between active/inactive conformations (Ellis and van der Vies 1991). This function is important in the interaction of Hsp90 and steroid receptors. The chaperone-bound receptor is stabilized in a state that has a high affinity for hormone, but that cannot bind DNA. Activation of

Hsp90 bound receptor in the presence of hormone requires ATP and involves ligand binding, release from hsp90, and a change in receptor conformation to a form that has high affinity for DNA (Csermely et al. 1998).

It has been shown that GR complex assembly is an ordered pathway consisting of GR complexes differing in subunit composition. There are various models of this complex formation. First Hsp40 binds to the GR. This interaction recruits Hsp70. Then Hsp70 binds to Hsp40 and to GR in the presence of ATP. Hydrolysis occurs. Then the GR complex with Hsp40 and Hsp70 forms a complex with Hop and Hsp90. Hop can bind to Hsp70 and Hsp90. Hsp40 and some Hsp70 and Hop leave the mature complex. When Hop leaves the complex an immunophilin can bind to Hsp90. Binding of p23 to Hsp90 occurs late in the pathway. The mature complex contains GR, Hsp90, Hsp70, immunophilin and p23. GR complexes are in steady-state cycle in which early, intermediate (Hsp90-Hop-Hsp40-Hsp70-GR) and mature complexes are present (Pratt and Toft 1997, Pratt et al. 2004b). See Figure 4.



- Figure 4. Model of Hsp90 chaperone complex formation. HOP binds to Hsp90 and Hsp70. Hsp70 binds to Hsp40 and to steroid receptor (SR). When Hop leaves the complex an immunophilin (IP) can bind to Hsp90. Some Hsp70 leaves the intermediate complex during assembly. Binding of p23 to Hsp90 occurs later.

It is known that steroid receptors envelop ligand in a hydrophobic space which is collapsed or altered in the absence of ligand. The receptor has to change its conformation to allow entry of the ligand. The binding to the GR is initiated with Hsp70 and Hsp40. Hsp90 binds to the ligand binding domain of the receptor and causes conformational changes that open a steroid-binding cleft. The site of Hsp90 binding to GR is unknown, but a short segment in the N-terminus of the ligand-binding domain is required for GR-Hsp90 heterocomplex assembly. The receptor bound Hsp90 must assume its ATP-dependent conformation and Hsp90 is then bound by p23, at which stage p23 binds to receptor bound Hsp90 in dynamic fashion and stabilizes the heterocomplex. Hop dissociates from the receptor-bound Hsp90 but it is still associated with Hsp70 (Pratt and Toft 2003, Pratt et al. 2004b).

Steroid receptors need to be directed to the nucleus so that they can regulate gene transcription. Nuclear import is governed by the stability of receptor heteromeric complexes (Defranco 2000). In the absence or presence of hormone nuclear import of PR was inhibited by sodium molybdate treatment (Yang and DeFranco 1996). Coexpression of NLS-Hsp90 with cytoplasmic GR mutant

led to complete nuclear localization of the receptor. This result suggested that Hsp90 possibly participates in nuclear-cytoplasmic shuttling (Kang et al. 1994).

AIMS OF THE PRESENT STUDY

The present work was undertaken to learn more about the cellular functions of Hsp90 in progesterone receptor action and to understand in cellular signaling how signaling proteins travel to their sites of action in the cell.

The specific aims of the study were:
1. to study the oligomeric complex between Hsp90 and progesterone receptors seen in vitro cell extracts existing in intact cells (I, IV)
2. to study the involvement of Hsp90 in the nuclear transport of progesterone receptor (II)
3. to investigate the determinants of the cytoplasmic location of Hsp90 and the strength of the cytoplasmic signal by comparing it with that of the nuclear transport signals of progesterone receptors (III)

MATERIALS AND METHODS

1. CELL CULTURE AND TRANSFECTION (I, III, IV)

COS cells were grown on glass coverslips and maintained in DMEM (LifeTechonologies, Gaitersburg) supplemented with 5% charcoal-stripped fetal bovine serum. Cells were transfected by lipofection using lipofectamine reagent (Life Technologies, Gaitersburg). 2 µg of DNA was diluted in 100 µl serum-free medium and 15 µl of Lipofectamine was diluted in 100 µl serum-free medium and these two mixtures were combined and incubated for 40 min at RT. Then the cells were rinsed with serum-free medium and 0.8 ml of serum-free medium was added to the tube containing DNA-liposome complexes and the mixture was added to the cells. The cells were then incubated with serum-free medium Optimem (Life Technologies), containing the DNA-liposome complexes for 24 h at 37 C. Next 1 ml of growth medium containing 10% serum was added without removing the transfection mixture and incubated for 2 h at 37 C. The medium was thus changed and the cells further incubated for 24 h.

2. ANTIBODIES (I, II, III, IV)

Mouse monoclonal antibody PR22, which recognizes both A and B components of PR (Sullivan et al. 1986) and polyclonal antibody αD, raised in rabbit against a synthetic peptide corresponding to residue 522-535 of chicken PR-B were used (Pekki et al. 1995). B10 antibody is a monoclonal antibody against human estrogen receptor (Shemshedini et al. 1992). Monoclonal antibody 7Dα is a chicken Hsp90-specific. It recognizes both free Hsp90 and the Hsp90 in the oligomeric complex (Schuh et al. 1985). The mouse monoclonal antibody PR6 raised against PR recognizes only B component of PR (Sullivan et al. 1986).

3. HISTOCHEMICAL TECHNIQUES (I, III, IV)

We used single (B10) and double (αD/PR22, αD/7Dα, PR6/αD) fluorescence labeling technique. After fixation the cells were washed in PBS for 10min incubated in 0.5% Triton-X-100 in PBS for 40 min at RT, and washed in PBS for 10 min. There after they were incubated in 10% normal rabbit or/and horse serum in PBS for 30 min. After the removal of excess serum, primary antibodies were applied to sections (B10 1:500, αD 1:200, PR22 and 7Dα 1 µg/ml at final concentration) and incubated overnight at 4 C. In double immunostaining αD/PR22, αD/7Dα and PR6/αD were mixed and applied to the sections simultaneously. The next day the cells were washed in PBS for 10 min, incubated with secondary antibodies biotynylated anti-mouse from goat, Amersham (1:400) and anti-rabbit-Flurescein (1:200) in PBS for 40 min. In double immunostaining the secondary antibodies were added to the section simultaneously. The cells were then washed in PBS for 10 min and incubated with rhodamine-labeled avidin D 1:100/150 in PBS for 30 min followed by washing in PBS for 10 min and mounting in fenylendiamin-glycerin (50mg fenylendiamin was diluted in 5 ml PBS, filtered and added to 45 ml glycerin pH 8.0; pH was adjusted to 9.0 with 0.5 M carbonate buffer).

4. CELL TREATMENTS (I, IV)

4.1. EGS and paraformaldehyde crosslinking

After transfection the cells were incubated on ice for 4 h and washed with ice-cold PBS. EGS (10 mM in PBS and 5% DMSO, freshly made) was added and incubated for 1 h. The reaction was

stopped by adding lysine (100 mM in PBS) and incubating 1 h. The cells were washed with ice-cold PBS and fixed with 4% paraformaldehyde on ice for 15 min. Paraformaldehyde crosslinking was carried out by adding paraformaldehyde directly to the cells at final concentration 1.5% and incubated for 5 min at 37 C. The cells were then washed in PBS and fixed in 4% paraformaldehyde.

4.2. H_2O_2 treatment
After transfection the cells were incubated on ice for 4 h and washed in cold PBS. 10 mM NaN_3 was added to the cells and incubated for 30 min on ice followed by incubation with 10 mM H_2O_2 in PBS for 30 min on ice. The cells were directly fixed in 4% paraformaldehyde on ice 15 min.

4.3. Molybdate treatment (A)
After transfection 30 mM sodium molybdate was added to the cells and incubated for 1 h at RT. Then the cells were incubated on ice for 4 h and washed with PBS containing 30 mM sodium molybdate for 10 min and fixed in cold 4% paraformaldehyde also containing 30 mM sodium molybdate.

4.4. Combined molybdate, H_2O_2 and EGS treatment
After transfection the cells were incubated with 30 mM sodium molybdate at RT and incubated for 1 h. The cells were incubated on ice for 4 h and washed in ice-cold PBS for 10 min. 10 mM NaN_3 was added and incubated for 30 min on ice. Then 10 mM H_2O_2 was added and incubated for 30 min at 0 C and washed in 30 mM sodium molybdate PBS for 10 min at 0 C. 10 mM EGS was added and incubated for 1 h at 0 C and the reaction was stopped with 100 mM lysine in PBS for 1 h at 0 C. The cells were then washed with 30 mM sodium molybdate for 10 min at 0 C and fixed in 4% paraformaldehyde containing 30 mM sodium molybdate for 15 min.

4.5. Cold treatment
After transfection cells were rinsed with DMEM supplemented with 5% charcoal-stripped FBS at 4 C and incubated on ice for 4 h. Then the cells were washed in PBS and fixed in 4% paraformaldehyde.

4.6. Molybdate treatment (B)
After 24 h incubation the transfected cells were washed in PBS 3 times and switched to phenol red-free DMEM plus 10% charcoal-stripped FBS and incubated for 8-10 h. The cells were then washed in PBS. 30 mM sodium molybdate and Lipofectamine (Life Technologies) was diluted 1:333 in phenol red-free and serum-free DMEM and the mixture was incubated for 40 min at RT, then added to the cells and incubated overnight at 37 C. The cells were then washed in PBS and fixed in 4% paraformaldehyde.

5. EXPRESSION VECTORS (I, III, IV)

HSP1 was constructed by amplifying a fragment of cHsp90 corresponding to amino acids 332-728 by PCR, using primers which incorporate an XhoI site at both the 5` and 3`ends. The PCR-ampilified HSP1 was inserted into pUK21 at the SmaI site. NLS1,2,3 was constructed by amplifying a fragment of hER corresponding to amino acids 8-315 by PCR, using primers which incorporate 5` Eco RI and XhoI sites and a 3` BglII site preceded by a stop codon. The NLS1,2,3 amplified by PCR was inserted into pUK21 at the SmaI site. The Eco RI-Bgl II fragment of NLS 1,2,3 was inserted into pSG5 at the corresponding sites. The HSP1-NLS1,2,3 construction was made by inserting HSP1 at the XhoI site in the NLS1,2,3. HSP1-NLS1,3 and HSP1-NLS3 and HSP1-NLS0 were constructed by replacing a Not I-Bgl II fragment (corresponding to amino acids

64-315) of HSP1-NLS1,2,3 with corresponding fragments of NLS deletion mutants of hER HE252G, HE244G and HE241G respectively.

Fusion proteins between chicken Hsp90 and chicken progesterone receptor were constructed by amplifying different portions of the cHsp90 by PCR and ligating them into the XhoI site of cPR (a pSR5 expression vector containing the whole cPR coding region). The XhoI site is located immediately before the first ATG codon. The wild-type chicken progesterone receptor (cPR21) and wild-type chicken Hsp90 expression vectors have been described (Turcotte et al. 1990, Tuohimaa et al. 1993). NLS-Hsp90 contain an NLS of human estrogen receptor (amino acids 253-303) in front of the coding sequence of chicken Hsp90 and has been characterized (Tuohimaa et al. 1993). PR35, NLS-deficient chicken progesterone receptor (amino acids 454-486 deleted) has been characterized by (Ylikomi et al. 1992).

6. IMMUNOBLOTTING OF HYBRID PROTEINS (III)

After the transfection cells were scraped from the Petri dishes and the proteins were extracted using M-PeR TM Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA) according to the manufacturer`s instructions, supplemented with complete miniprotease inhibitor (Roche Diagnostics, Mannheim, Germany). Samples were mixed with 1 vol. 2 x SDS/sample buffer and boiled for 5 min. Samples were dissolved in 6.5% polyacrylamide slab gels containing 0.1% SDS and transferred to nitrocellulose membrane with electrophoretic transfer apparatus (Mini Trans-blot, Bio-Rad). The membranes were presaturated with 5% skimmed milk powder in Tris/NaCl/Pi/Tween (50 nM Tris pH8.0, 0,9% NaCl, 0.05% Tween) and then incubated with the primary antibody PR 22 (1,0 $\mu g.mL^{-1}$) in Tris/NaCl/Pi/Tween containing 1% skimmed milk powder at 4 C overnight. After washing with Tris/NaCl/Pi/Tween the membranes were incubated with peroxidase-conjugated goat anti-(mouse Ig) Ig (Cappel, West Chester, PA, USA) diluted 1:40000 in Tris /NaCl/Pi/Tween with 1% skimmed milk powder. The peroxidase was visualized using the enhanced chemiluminescence method according to the manufacturer`s instructions (Amersham).

7. ANIMALS AND TREATMENTS (II)

Two-week-old white Leghorn chicks were used. They were treated with 17-beeta-estradiol (Sigma) dissolved in propylenglycol, 1 mg/d per animal, for 1 week followed by a withdrawal period for another week. After the withdrawal period, the animals received hormone injections for 4 days and they were sacrificed after 24 h after the last injection by cervical dislocation. Magnum parts were dissected immediately and stored in liquid nitrogen.

8. CYTOSOLIC EXTRACTION AND TREATMENTS (II)

Oviduct samples were homogenized in 3 volumes of in vitro transport buffer (20 mM N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM ethylene glycol-bis beta-amino-ethyl ether-N,N,N,N-tetraacetic acid, 2 mM dithiotreitol, both containing a mixture of protease inhibitors. Then the mixture was centrifuged 100000 g for 1 h at 4 C. The supernatant fraction obtained was used as cytosol and was either used as such or treated with 0.02 M sodium molybdate or 0.3 M potassium chloride for 2 h at 4 C. KCl-treated cytosols were dialyzed overnight at 4 C against transport buffer.

9. ISOLATION OF NUCLEI FROM HeLa CELLS (II)

The cells were cultured in DMEM (Life Technologies, Gaitersburg) in tissue culture flasks (5 to 50 x 10^6 cells). The medium was removed and the cells were placed on ice. Each flask was washed twice with 5 ml ice-cold PBS. Then PBS was removed and 5 ml of fresh PBS was added. The cells were gently dislodged from the plastic surface by scraping with a glass rod connected to a rubber policeman. Cells were collected and centrifuged for 5 min at 500 g at 4 C. The supernatant was removed completely. The cell pellet was loosened by vortexing at half maximal speed for 5 sec prior to the addition of 4 ml NP-40 lysis buffer (10 mM Tris.Cl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40). Vortexing was continued as the buffer was added. Once the lysis was completely added, the cells were vortexed at maximum speed for 10 sec. The lysed cells were incubated on ice for 5 min and centrifuged 5 min at 500g at 4 C. The supernatant was removed. The nuclear cell pellet was resuspended in 4 ml NP-40 lysis buffer by vortexin as described before and centrifuged for 5 min at 500g at 4 C. The supernatant was discarded and the pellet was resuspended in 200 µl glycerol storage buffer (50 mM Tris hydrochloride, pH 8.3, 40% glycerol, 5 mM magnesium chloride, 0.1 mM ethylenediamine-tetraacetic acid) by gently vortexing. The resuspended nuclei were frozen in liquid nitrogen. The nuclei are stable in liquid nitrogen for more than 1 year.

10. NUCLEAR TRANSPORT (II)

10.1. In vitro assay of nuclear transport with permeabilized HeLa cells

HeLa cells were grown on glass cover slips in Dulbecco modified Eagle Medium/F12 with 5% fetal bovine serum for 24 to 48 h. The cells were washed twice in transport buffer. Then 40 µg/ml digitonin in transport buffer was added to the cells and incubated for 5 min. The cells were washed twice in transport buffer. The final transport mixture contained 50% (vol/vol) of either untreated or KCl-treated cytosol, 0.02 M sodium molybdate, 1 mM adenosine triphosphate (ATP), 5 mM creatine phosphate and 20 U/mL of creatine phosphokinase diluted in transport buffer and was added to the cells and incubated for 30 min at 30 or 4 C. In some experiments progesterone was added as a final concentration of 1 µM to the transport mixture before incubation at 30 C. The cells were then washed twice in transport buffer and fixed in ice-cold methanol for 10 min RT. Cells were washed in ice-cold PBS twice and thereafter they were incubated in 10% normal horse serum for 30 min. After removal of serum primary antibodies PR22 and 7Dα was 1 µg/mL were added to the cells overnight at 4 C. Then the cells were washed twice in PBS and secondary antibodies 1:1000 were added and incubated for 30 min. After washing twice in PBS the cells were incubated with prepared avidin-biotin-peroxidase complex for 30 min. Peroxidase was visualized using 0.025% 3.3-diaminobenzine-HCl (Sigma Chemicals, USA) and 0.03% hydrogen peroxide in 0.5 M Tris-HCl saline as substrate and the reaction was terminated in 0.5 M Tris-HCl buffer pH 7.6. The sections were then dehydrated and mounted in Entellan (Merck, Germany).

10.2. In vitro assay of nuclear transport with isolated nuclei of HeLa cells

Isolated nuclei (7 x10^5 to 1 x10^6 nuclei /reaction) were incubated for 10, 20 or 30 min at 30 C or at 4 C for 30 min with transport mixture (25-35% vol/vol cytosol, 5 mM ATP, 5 mM creatine phosphate and 20 U/mL of creatine phosphokinase diluted in transport buffer). Then the samples were centrifuged at maximum speed for 20 sec. The supernatant was taken and the nuclei were washed in 0.1% Triton-X-100 in PBS and centrifuged. The supernatant was discarded and the samples were resuspended in 2 x SDS-PAGE sample buffer and boiled for 5 min. The resulting nuclear fraction was analyzed by immunoblotting (Pekki et al. 1995). The pellet was taken in 1:5 of

vol sample buffer (62.5 mM Tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% beta-mercaptoethanol, 0.05% bromophenol blue) and boiled for 5 min. The proteins were dissolved in 12% polyacrylamide slab gels containing 0.1% SDS (Laemmeli 1970) and transferred to nitrocellulose membrane with an electrophoretic transfer apparatus (Mini Trans-blot, BioRad, Richmond, Ca). The membranes were saturated with 5% bovine serum albumin (BSA) in TBS (50 mM Tris, 0.9% NaCl, pH 8.0) and then incubated with primary antibody 7Dα at 4 C overnight. After washing the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG diluted 1:10000 in TBS containing 1% BSA. The peroxidase was visualized using ECL method according to manufacturer's instructions (Amersham).

11. CONFOCAL MICROSCOPE (I, III, IV)

A confocal microscope, Bio-Rad MRC1024, connected to a Zeiss Axiovert 135M (Carl Zeiss Jena GMbH, Göttingen, Germany) inverted microscope was used. The laser was argon/krypton (American Laser Corporation, Salt Lake City, UT, USA). Fluorescein isothiocyanate-stained samples were imaged by excitation at 488 nm with a 506-541 nm bandpass emission filter. TRITC-stained excitation was imaged at 568 nm with a 585nm long-pass emission filter. The different fluorophores were imaged separately to obtain images with no excitation/emission wavelength overlap; the separate images were later merged into a single image.

RESULTS

1. OLIGOMERIC COMPLEX BETWEEN PROGESTERONE RECEPTORS AND HSP90 (I, IV)

In cell extracts all steroid receptor molecules are found as oligomeric complexes between Hsp90 and other proteins (Dougherty et al. 1984, Catelli et al. 1985, Kost et al. 1989, Smith et al. 1990, Johnson et al. 1994). Immunohistochemical studies have shown that Hsp90 and steroid receptors are located in different cell compartments (Pekki 1991, Tuohimaa et al. 1993). We studied whether and to what extent steroid receptors and Hsp90 are associated in vivo in intact cells. Cos-7 cells were transfected with wt-PR (whole length B-form of the chicken PR cDNA), PR35 (nuclear localization signal-deficient chicken PR, 454-486 amino acids deleted, 2 of the 4 nuclear localization proto-signals have been deleted), wt-Hsp90 (wild-type chicken Hsp90) and NLS-Hsp90 (an Hsp90 molecule to which a nuclear localization signal has been added). The receptor was detected using double immunohistochemistry. We used antibody polyclonal antibody αD, which can distinguish between oligomeric and dissociated form of the PR. The epitope of the antibody is located in a domain which is hidden in the oligomeric form (domain D of the PR) and the antibody recognizes only the dissociated form in vitro. We also used antibodies PR22 and PR6 that recognize both forms of PR.

Due to the dynamic interaction between receptors and receptor-associated proteins it is difficult to study the interaction in vivo in oligomeric complex (Smith and Toft 1993). In our study the dynamic interaction was stabilized using molybdate, cold treatment, by in vivo crosslinking by bifunctional crosslinker EGS, by paraformaldehyde, and by oxidative stress. Cold and molybdate treatments are known to inhibit nuclear translocation and result in cytoplasmic accumulation of the steroid receptors (Guiochon-Mantel et al. 1991, Rexin et al. 1991). In our study cold and molybdate treatment of the wt-PR transfected cells resulted in cytoplasmic accumulation of PR as detected with PR22/PR6. The nuclear periphery was also stained. αD showed intense nuclear staining in treated cells with faint cytoplasmic staining. Co-transfection of Hsp90 with wt-PR did not alter the staining pattern. Hsp90 was seen to localize exclusively in the cytoplasm. In vivo crosslinking and oxidative stress did not affect the immunostaining with αD and thus were inefficient in demonstrating oligomeric receptor form. This suggests that in the nucleus most of the receptor molecules are in dissociated form and in the cytoplasm the portion of the cytoplasmic receptor seen as an oligomeric complex is small.

When cytoplasmic PR mutant expression was studied by PR22 and αD identical cytoplasmic staining was seen. We also transfected cytoplasmic mutant of PR with wild-type Hsp90. When the cells were stained with PR22 and αD, considerably lower staining intensity was seen with αD, which would suggest that portion of the transfected Hsp90 interacts with PR. Cotransfection of NLS-Hsp90 with wild-type PR did not change the intensity of αD staining, suggesting that PR is in dissociated form in the nucleus and in the cytoplasm. The association of Hsp90 with PR in vivo was also studied by forcing the Hsp90 and steroid receptors in the same cell compartment coexpressing cytoplasmic PR mutant and NLS-Hsp90 and immunohistochemistry using αD antibody. When cytoplasmic PR mutant PR35 was co-expressed with the nuclear form of Hsp90 (NLS-Hsp90), a portion of PR was co-translocated into the nucleus. This suggests that steroid receptors are associated with Hsp90 in intact cells, but the Hsp90-asociated receptor pool represents only a small portion of the receptors.

2. CYTOPLASMIC ANCHORING OF HSP90 (III)

There are conflicting views about the subcellular localization of Hsp90 (Gasc et al. 1984, Lai et al. 1984, Perdew et al. 1993, Pekki et al. 1994). Immunohistochemical studies have shown that both endogenous and transfected Hsp90 are located in the cytoplasm and steroid receptors are located in the nucleus (Pekki 1991, Tuohimaa et al. 1993). Proteins destined for the nucleus contain specific nuclear localization signals (NLS) that mediate their nuclear transport (Dingwall and Laskey 1986). We wanted to study the determinants of the cytoplasmic localization of Hsp90.

In Drosophila maternal morphogen dorsal has been shown to accumulate predominantly in the cytoplasm because it has an anchoring sequence at the C-terminus (Rushlow et al. 1989, Hough et al. 1997) thus we wanted to study whether Hsp90 has sequences which dictate its cytoplasmic localization. Sequences that dictate the nuclear localization of proteins were studied by constructing hybrid proteins between cytoplasmic proteins and potential nuclear localization signal containing polypeptide. Analogously we studied the sequences responsible for cytoplasmic localization by constructing hybrid molecules with Hsp90 sequences and nuclear protein (PR). We first constructed hybrid molecules in which either the N-terminal (cPR-HSP2) or C-terminal (cPR-HSP1) half of Hsp90 was fused with a nuclear protein, wt-PR. Then the molecules were transfected into the Cos cells and studied by immunohistochemistry by PR6 and PR22 antibodies. cPR-HSP1 showed cytoplasmic staining in the presence and absence of hormone. The cPR-HSP2 showed nuclear staining with weak cytoplasmic staining. In the presence of ligand nuclear staining was seen.We also constructed a series of hybrid molecule containing C-terminal amino acids of the Hsp90 in front of wt-PR to ascertain where the sequence of the cytoplasmic signal is localized. The constructs showed only nuclear staining. This indicated that the sequence which prevents Hsp90 from translocating into the nucleus is between 33 and 664 amino acids at the C-terminal end of the molecule.

We also wanted to study the strength of the cytoplasmic anchoring and to compare it with NLS of ER. We made four chimeric molecules between C-terminal half of Hsp90 and fragments of ER which carry different deletions of proto-NLS. The constructs were transfected into Cos cells and stained with anti-ER Ig. Only cytoplasmic staining was seen when HSP1-NLS0 (all of the proto-NLS signals were deleted) was transfected. Corresponding ER mutant HE17 was seen equally in the nucleus and cytoplasm. HSP1-NLS3 (which has one proto-NLS) showed cytoplasmic staining and corresponding ER mutant HE 16 showed equal staining in the nucleus and cytoplasm. HSP1-NLS1,3 (which has two proto-NLSs) and HSP1-NLS1,2,3 (which has three proto-NLSs) also located exclusively in the nucleus similarly to their corresponding ER mutants HE39A and HE39. Thus the hybrid molecule was exclusively cytoplasmic when weak NLS was used and exclusively nuclear with strong NLS.The findings suggest that Hsp90 contains a cytoplasmic anchoring sequence in the C-terminal half of the molecule which retains it as a cytoplasmic protein.

3. HSP90 AND NUCLEAR TRANSPORT OF PROGESTERONE RECEPTOR (II)

Hsp90 is a chaperone protein and has been shown to associate with steroid receptors soon after receptor synthesis (Dalman et al. 1990, Dalman et al. 1991, Whitfield et al. 1995). The application of a cell permeabilization technique has made a major contribution to in understanding nuclear protein import and export. By the use of detergent digitonin cell membranes rich in cholesterol can

be permeabilized selectively. Permeabilization allows the replacement of an endogenous cytosol with an exogenous one (Liu et al. 1999).

We studied nuclear transport by permeabilized HeLa cells and replaced the cytosol with chicken oviduct cytosol. We used PR22 antibody, which recognizes chicken PR. PR was seen to accumulate into the nucleus when a cytosol-containing liganded PR was incubated with permeabilized cells. When molybdate-stabilized PR was allowed to bind ligand and then incubated with permeabilized cells, no nuclear accumulation was seen. When molybdate was added after the ligand, PR was seen to accumulate into the nucleus. We demonstrated that the stabilized oligomeric complex of PR could not be transported into the nucleus. When purified HeLa cell nuclei were incubated with cytosol in the presence of ATP, PR was seen to be transported into the nucleus, whereas Hsp90 remained in the cytosol. Unliganded PR accumulated in the nucleus when it was salt dissociated from Hsp90 but Hsp90 remained in the cytosol. The presence of molybdate inhibited nuclear accumulation and in the absence of ATP no nuclear accumulation of PR was seen. The results indicate that PR in its oligomeric complex with Hsp90 cannot be transported into the nucleus.

DISCUSSION

1. OLIGOMERIC COMPLEX

1.1. Association of Hsp90 and steroid receptors in vitro

The complex between steroid receptors and Hsp90 can be demonstrated in cell-free lysates (Dougherty et al. 1984, Catelli et al. 1985, Kost et al. 1989, Smith et al. 1990, Johnson et al. 1994). In vitro all steroid receptor molecules have been shown to be associated with Hsp90 (Pekki et al. 1995). The studies are mainly based on in vitro studies because dynamic interaction between receptor-associated proteins and receptors makes it difficult to study the oligomeric complex in living cells. There are many studies on the in vitro assembly of the oligomeric complex (Pratt and Toft 1997). The in vitro assembly of oligomeric complex requires ATP, Mg^{2+}, K^{+} and near physiological temperature (Hutchison et al. 1992b, Smith et al. 1992, Hutchison et al. 1994). Several proteins in addition to Hsp90 are present in untransformed steroid receptor complexes like Hsp70, p23, Hsp40, Hop (Pratt and Toft 2003). The untransformed complex is the 8-9S complex and the transformed, dissociated form is the 4S complex (Gorski et al. 1968, Baulieu et al. 1971, Jensen and DeSombre 1972, Jensen and DeSombre 1973, O'Malley and Means 1974). Salt causes the dissociation of the 9S complex to 4S complex (Baulieu and Jung 1970, Baxter and Tomkins 1971, Beato and Feigelson 1972, Jung and Baulieu 1972). It has been suggested that oligomeric complex may be an in vitro phenomenon brought about by the artifactual association of various proteins, enzymes, other factors as a result of cell ruption and liberation with hydrophobic regions into aqueous phase during cytosol preparation (Grody et al. 1982, Pekki et al. 1994, Freeman et al. 1996, Ylikomi et al. 1998).

A stable oligomeric complex can be isolated from cells not exposed to hormone (Smith and Toft 1993). PRs in cytosols prepared from chicken oviduct were shown to migrate as 8-9S form in low-ionic strength sucrose gradients and 8-9S form is converted into 4S form at high-ionic strength (Sherman et al. 1970). When purified PR as well as other steroid receptors (GR, ER, MR) are incubated with reticulocyte lysate, receptors are converted back to Hsp90-bound form (Toft and Gorski 1966, Grody et al. 1982, Smith et al. 1990). The mechanism whereby the lysate reconstitutes the oligomeric complex from the purified receptor is unknown. Using phase partitioning and hydrophobic interaction chromatography it has been demonstrated that both Hsp90 and steroid receptors are extremely hydrophobic. Thus steroid receptors are good targets for Hsps whose function is to cover the hydrophobic regions of other proteins. It has been shown that the LBD of GR and ER is extremely hydrophobic and are good targets for Hsp interaction whereas the LBD of retinoic acid receptors are more hydrophilic and are less good targets for Hsp interaction. The difference in the hydrophobicity can thus explain why steroid receptors can be seen as oligomeric complexes in tissue extracts while retinoic acid receptors cannot.

1.2. Association of Hsp90 and steroid receptors in vivo

Most immunohistochemical studies, however, have shown that Hsp90 is a cytoplasmic protein whereas PR has been shown to locate in the nucleus (Pekki 1991, Tuohimaa et al. 1993). For instance, in chicken oviduct cells PR was nuclear in the presence and absence of ligand, while Hsp90 was cytoplasmic (Pekki 1991). Here we have shown that, when PR and Hsp90 were transfected into HeLa cells, Hsp90 was in the cytoplasm and PR in the nucleus. Due to the dynamic interaction between receptor-associated proteins and receptors, it is difficult to study the oligomeric complex in living cells. Thus in vivo crosslinking has been used to stabilize the oligomeric complex in vivo (Rexin et al. 1991). By using in vitro crosslinking there is evidence that cytoplasmic receptor forms can interact with Hsp90 since GR can be crosslinked to Hsp90 in intact cells (Rexin et al. 1991, Alexis et al. 1992). There is also evidence that ER can be crosslinked in vivo with the

Hsp90 in intact cells in the absence of ligand (Segnitz and Gehring 1995). There is also a study where an unidentified 50-kDa protein but not Hsp90 was associated with ER by in vivo crosslinking (Rossini and Camellini 1994) whereas in another study ER was crosslinked with Hsp90 (Segnitz and Gehring 1995). The reason for the difference between these two studies is unknown. In Segnitz and Gehring`s work, cells were incubated for 2h on ice before the crosslinkers were added (Segnitz and Gehring 1995). Cold prevents nuclear transport and shuttling and nuclear proteins become cytoplasmic (Guiochon-Mantel et al. 1991). It is possible that the authors studied the nuclear receptor that accumulated in the cytoplasm

In our studies the association of progesterone receptors and Hsp90 in vivo was examined by using antibody αD, which can distinguish between oligomeric and dissociated forms. It recognizes the dissociated form of the receptor in vitro, since the epitope of the antibody is located in domain D of the PR, which is hidden in the oligomeric complex (Pekki et al. 1995). We studied whether PR and Hsp90 can associate provided they are present in the same cell compartment. We co-expressed cytoplasmic mutant of PR with cytoplasmic Hsp90 or wt-PR and NLS-Hsp90 and utilized different agents and treatments to stabilize the Hsp90-PR complex in vivo. Crosslinking the cells in vivo by using EGS and paraformaldehyde we were unable to demonstrate an oligomeric complex since the epitope of αD was exposed in both cases. Increased oxidative status of the cytoplasm, that has been shown to render the oligomeric complex more stable (Tienrungroj et al. 1987), was also unable to affect the αD-staining. Overexpressing Wt-Hsp90 with the cytoplasmic form of PR decreased the αD-staining, but did not express the nuclear form of Hsp90 with the WT-PR. This suggests that some of cytoplasmic receptors could interact with the Hsp90. The failure to detect the oligomeric complex after stabilizing the complex in vivo suggests that the fraction of the PR that interacts with the Hsp90 in vivo is small.

Molybdate and cold treatment was also used to stabilize the oligomeric complex. We studied the oligomeric complex by transfecting wt-PR into the cells and stabilizing the complex with cold treatment or molybdate. Molybdate and cold treatment resulted in cytoplasmic accumulation of PR. αD stained predominantly nuclear PR, whereas the antibody that can recognize both receptor forms stains both the nuclear form and cytoplasmic form of the receptor. These findings suggest that a portion of the cytoplasmic receptor is seen as an oligomeric complex, but in the nucleus most receptors were in dissociated form.

The existence of the oligomeric complex has been supported by transfection studies in which Hsp90-NLS was coexpressed with a cytoplasmic mutant of the receptor and the cytoplasmic receptor form was seen to co-translocate into the nucleus by the nuclear Hsp90 (Tuohimaa et al. 1993, Kang et al. 1994). This was also seen in our experiments. It was concluded that because cytoplasmic receptors are co-transported into the nucleus by NLS-Hsp90, wild-type steroid receptors should co-transport cytoplasmic Hsp90 into the nucleus. However, it should be noted that wildtype PR cannot significally change the location of wildtype Hsp90, indicating that without a heterologous NLS wild-type Hsp90 is a cytoplasmic protein which is not affected by nuclear receptors. Moreover, the magnitude of the co-translocation is small, suggesting that the association is not very efficient.

These results suggest that the major portion of the receptors, in vivo, is not associated with the Hsp90 and that the oligomeric complexes seen in cell extracts may be formed during cell fractionation. This is further supported by the fact that nonliganded receptor monomers can be reconstituted to the oligomeric complex in various cell lysates (reticulocyte, mouse L cell, insect cell, plant cell) and homogenates (Smith et al. 1990, Tuohimaa et al. 1993, Stancato et al. 1996). It may be suggested that the majority of PRs in the nucleus are associated with chromatin proteins.

During homogenisation steroid receptors (hydrophobic molecules) are released into aqueous milieu, which initiates the formation of the stable oligomeric complex which can be seen as a transient complex in vivo (Smith et al. 1990, Stancato et al. 1996). In intact cells in vivo the oligomeric complex can be seen as a transient complex after receptor synthesis and possibly also in the cytoplasm during receptor shuttling, whereas in cell homogenate highly hydrophobic steroid receptors and Hsp90 form a stable oligomeric complex that is an in vitro phenomenon.

2. SUBCELLULAR DISTRIBUTION OF PR AND HSP90

2.1. PR and ER

Early studies of steroid receptor localization by indirect immunofluoresence showed them to disperse throughout the nucleus and to be excluded from nucleoli (Warembourg et al. 1986, Yamashita 2001). The construction of receptor fusions with jellyfish fluorescent proteins made it possible to examine receptor localization and movement in the nuclei of living cells (Htun et al. 1996). There are many studies on the subcellular localization of ER and PR. Their localization has mainly been studied by immunohistochemistry. In normal breast and benign breast disease the progesterone receptor was localized in the nucleus in the ductal epithelial cells and hyperplastic epithelium. In uterus PR was shown by immunostaining to localize in the nucleus in stromal and glandular epithelial cells and in myometrium of smooth muscle cells (Press and Greene 1988). In the oviduct of estrogen-primed chicken both PR isoforms were localized in the nuclei of the cells (Syvala et al. 1996). In the absence of ligand cPR-B transfected into HeLa cells PR was localized in the nucleus (Ylikomi et al. 1992). GFP-PR-A was localized in the nucleus whereas GFP-PR-B was localized in the nucleus and cytoplasm (Kawata et al. 2001). In the chicken oviduct estrogen receptor was found in the nuclei of glandular epithelial cells and progesterone receptor was found by immunohistochemistry in the nuclei of glandular and epithelia, stroma, smooth muscle cells and mesothelium (Isola et al. 1987). In cultured human breast carcinoma cells ERα was observed only in the nucleus and ERα was localized in the nucleus when the cells were transfected with ERα cDNA (Ylikomi et al. 1992). GFP-ERα is present in the nucleus (Kawata et al. 2001). Hiroi et al also demonstrated ligand-independent nuclear localization of both ER isoforms (Hiroi et al. 1999). We studied the localization of PR by transfecting wild-type PR into Cos cells and analyzed the results by immunofluorescence. PR showed nuclear staining with PR22 and αD antibodies. When PR mutant in which two of the four NLSs have been deleted was transfected, cytoplasmic staining was seen with PR22 and αD. Our results concur with the studies of other authors. We used a confocal microscope in our studies. The advantages of a confocal microscope and fluorescence staining are that it is effective in discriminating out-of-focus information, vertical sectioning, enabling visualizing structures in three dimensions, volumetric analysis, analyzing colocalization, multiple-labeling.

2.2. Hsp90

Most immunohistochemical studies with light and electron microscope using different fixation techniques have shown that Hsp90 is a cytoplasmic protein, but there are reports that Hsp90 has both cytoplasmic and nuclear distribution in rabbit uterine cells (Gasc et al. 1984, Gasc et al. 1990, Perdew et al. 1993). Intranuclear localization of the Hsp90 may vary, but its association with the nucleoli and with the perichromatin ribonucleoprotein fibril have been reported (Owens-Grillo et al. 1996). We studied the localization of Hsp90 by transfecting wild-type Hsp90 into the Cos cells and cytoplasmic staining was seen. Proteins contain specific nuclear localization signals (NLSs) and nuclear export signals (NESs) that are responsible for active nuclear targeting and the export of proteins (Gorlich and Mattaj 1996, Nigg 1997). Hsp90 lacks NLS in its structure. There are different mechanisms for rendering proteins cytoplasmic. Membranes are involved in cytoplasmic

anchoring. Membrane-associated gyanylate kinase homologs ` domain HOOK is required for localization of protein to the cell membrane (Rushlow et al. 1989, Hough et al. 1997). Masking of NLS also renders protein cytoplasmic like NF-κB, a transcription factor which is in unstimulated cells cytoplasmic protein by binding to inhibitor protein which masks NLSs (Latimer et al. 1998). NES (nuclear export signal) is a mechanism translocating proteins from the nucleus into the cytoplasm. NES proteins bind exportins like CRM1 and passage is unidirectional (Yang et al. 1998). We studied the determinants of the cytoplasmic location of Hsp90 by constructing hybrid molecules between PR and parts of Hsp90 and demonstrated that Hsp90 contains a sequence in its C-terminal half which is responsible for its cytoplasmic location. We also studied the strength of the cytoplasmic signal by constructing four chimeric molecules between C-terminal half of Hsp90 and ER with different numbers of proto-NLSs. Deletion of the proto-NLSs one by one in the ER gradually changed the subcellular localization from nuclear to cytoplasmic. We suggest that Hsp90 contains a cytoplasmic localization sequence and does not function as NES because gradually increasing the strength of the NLS would have gradually changed the equilibrium of nuclear accumulation and nuclear export. It is possible that sequences in the C-terminus interact with cytoplasmic structures and that the interaction can be influenced by strong NLS.

3. ROLE OF HSP90 IN STEROID RECEPTOR ACTION

In the late 60`s and in the early 70`s the sucrose gradient centrifugation technique of various steroid hormone receptors established the existence of 8-9S and 4S forms of steroid receptors. However, the nature of these receptor forms was not established at that time (Gorski et al. 1968, Baulieu et al. 1971, Jensen 1991). It was suggested that the transformation of the form 8S to 4S represents an activation of the receptor form a cytoplasmic, inactive (non-DNA-binding) form into an active (DNA-binding) nuclear form (Jensen et al. 1968). In 1984 highly purified 8-9S PR was shown to contain two 90-kDa proteins with one molecule of PR (Renoir and Mester 1984). The 90-kDa was later identified as Hsp90 and was shown to be associated with all the steroid receptor receptors in vitro in cell homogenates (Smith and Toft 1993). Hsp90 was then considered a kind of corepressor (before the term corepressor was generally established) since it inhibited receptor-DNA interaction (Kost et al. 1989, Onate et al. 1991). Later other functions for the Hsp90 in the oligomeric complex were identified. During late 80`s it was discovered that Hsp90 interaction stabilizes the steroid receptor since Hsp90-free steroid receptors could not bind their ligand (Bresnick et al. 1989). The discovery that the oligomeric receptor form can be reconstituted in a reticulocyte lysate from purified steroid receptors has resulted in the establishment of current knowledge of the role of Hsp90 and other receptor interacting proteins as a part of the chaperone machinery which connects receptor-Hsp90 heterocomplexes to the post transcriptional modification of receptor molecules and protein trafficking pathways (Pratt and Toft 1997).

3.1. Synthesis and processing of progesterone receptors

Molecular chaperones are defined as proteins that bind to and stabilize unstable proteins by binding and release. They facilitate folding, oligomeric assembly, transport to a particular subcellular compartment (Ellis and van der Vies 1991). Hsp90 has been studied most extensively in relation to steroid receptors. Hsp90 has been shown to facilitate proper folding, trafficking, transcriptional activation and turnover of the steroid receptors. There has been argument over the nature of the interaction between the Hsp90 and steroid receptor in vivo (Pratt and Toft 1997).

Hsp90 binds to the receptor in a co-ordinated action of several proteins that function as chaperone machinery. Hsp90 plays a role as a central organizer of the chaperone complex which aid

maturation of the receptors. This is an ATP-dependent process and assembles receptor-Hsp90 heterocomplexes. When immunopurified GR is incubated with rabbit reticulocyte lysate in the absence of hormone, receptor is assembled into a heterocomplex with Hsp90. The minimal system for efficient assembly of the heterocomplex in reticylocyte lysate consists of five purified proteins: Hsp90, Hsp70, Hop, Hsp40, p23 (Pratt and Toft 2003). For GR, Hsp90 must bind to LBD so that it can have high-affinity steroid-binding activity. If Hsp90 dissociates in the absence of hormone, the GR hormone binding domain collapses and loses its steroid binding ability (Bresnick et al. 1988, Bresnick et al. 1989). Hsp90 and Hsp70 operate together as a part of the chaperone machinery and open a hydrophobic steroid-binding cleft in a properly folded state of the GR. Steroid binding closes the cleft and GR loses the ability to form persistent complexes with Hsp90 (Pratt and Toft 1997, Morishima et al. 2001).
We studied if Hsp90 and PR can associate in vivo and showed that when PR and Hsp90 are present in the same cell compartment they are associated in vivo in the cytoplasm but present only a small pool of all PR molecules. This result supports that Hsp90 is a chaperone and interacts transiently with a nuclear receptor.

3.2. Cytoplasm-nuclear transport

Molecules enter the nucleus by two mechanisms: passive diffusion and active transport (Guiochon-Mantel et al. 1991). Small molecules 40-60kDa enter by passive diffusion and larger molecules require ATP so that they can enter the nucleus (Peters 1986, Richardson et al. 1988). Nuclear localization signals (NLSs) are responsible for targeting proteins into the nucleus (Dingwall and Laskey 1986). The passage through the nuclear pore is very rapid. Nucleocytoplasmic signaling of proteins is based on anchoring and release and masking and unmasking of NLSs (Nigg 1990). In the absence of hormone GR is cytoplasmic but when it becomes bound by ligand it shifts to the nucleus (Yang and DeFranco 1994, Yang and DeFranco 1996). Steroid-dependent translocation is rapid. The movement can be divided into 1) movement from the cytoplasm into the nuclear pore 2) transport across the nuclear pore complex 3) movement in the nucleus 4) dynamic exchange of transcription factors between chromatin and the nucleoplasmic compartment (Pratt et al. 2004a).

Most of the studies on the effects of Hsp90 on receptor function in vivo have focused on the GR. It has been shown that nuclear targeted Hsp90 shifts GR into the nucleus. Hsp90 does not have NLS and it seems likely that the presence of Hsp90 in the nucleus is due to the fact that it is carried in by an NLS-containing client protein (Yang and DeFranco 1994, Yang and DeFranco 1996). Hsp90-dependent trafficking occurs along the cytoskeletal tract (Pratt et al. 1999). The effects of the heterocomplex machinery on receptor trafficking have been studied indirectly using Hsp90 inhibitors, but there is no evidence that Hsp90 is directly involved in nuclear transport (Pratt et al. 2004b).

We studied the involvement of Hsp90 in the nuclear transport of PR by using digitonin permeabilized cells and purified nuclei in in vitro transport systems. We demonstrated that unliganded PR, which was salt dissociated from Hsp90, was transported into the nucleus. Hsp90 remained in the cytosol. When the oligomeric complex of the PR was stabilized with molybdate no nuclear accumulation was observed. This was also shown by intact cells when molybdate was added to the cells by liposomes. When we added molydate after ligand, PR accumulated in the nucleus. Our results suggest that Hsp90 assists the chaperoning process of PR preceding nuclear transport of the receptor and that the oligomeric complex is short-lived, dissociating before or during the nuclear transport.

3.3. Role in the nucleus

Virtually nothing is known of how molecules move within the nucleus (Phair and Misteli 2000). Steroid receptors constantly pass into and out of the nucleus (Guiochon-Mantel et al. 1991). In the absence of hormone receptor may be predominantly cytoplasmic or nuclear, depending on the receptor (Defranco et al. 1995). Many coactivators and corepressors have been identified to be involved in nuclear receptor-regulated transcription (Torchia et al. 1998). Steroid receptors do not have DNA-binding activity when they are in a complex with Hsp90 (Scherrer et al. 1990). Many LBD deletion mutants of steroid receptor that result in a constitutively active receptor also do not interact with Hsp90. Thus it has been proposed that Hsp90 functions as a corepressor by inhibiting the interaction of the receptor with the target gene (Cadepond et al. 1991, Scherrer et al. 1993). It has also been proposed that Hsp90 heterocomplex assembly may be involved in the termination of the transcriptional action when steroid dissociates from DNA-bound receptors in intact cells (Scherrer et al. 1990). Ligand binding causes a profound change in the structure of the LBD and also transconformation of the helix 12, which closes like a lid on the entrance of the ligand binding pocket (Renaud et al. 1995, Wurtz et al. 1996). This results in a decrease in the surface hydrophobicity of steroid receptors and the release of Hsp90 (Hansen and Gorski 1986, Fritsch et al. 1992). However, not all constitutively active receptor mutants are devoid of the ability to interact with the Hsp90, since a point mutation in the helix 12 of the LBD can generate active ER, which can interact with Hsp90 (White et al. 1997).

It has been proposed that unliganded nuclear receptors may also have the capacity to interact with chromatin proteins (Liu and DeFranco 1999). Non-liganded ER has been detected as complex with chromatin proteins (Rossini and Camellini 1994). PR has been shown to interact with chromatin constituents like high mobility group chromatin protein 1 and 2 (Boonyaratanakornkit et al. 1998). Our results suggest that steroid receptors are not associated with Hsp90 in the nucleus. It is thus possible that interaction with the chromatin proteins protects the extremely hydrophobic LBD of the steroid receptors. When this interaction is disturbed during tissue homogenization, receptor is released into an aqueous phase and is a good target for Hsp90 interaction.

3.4. Cytoplasmic effects of steroid receptors

Estrogens and progestins have been shown to cross-talk with a number of other signaling pathways, including cyclic AMP, Ca-calmodulin, G-protein-coupled receptors and MAP kinase pathway (Ballare et al. 2003). ERα and ERβ have been shown to influence AP-1 dependent transcription by cytoplasmic signal transduction pathways in the cytoplasm. The effects are mediated by interactions at the membrane or cytoplasmic level (Bjornstrom and Sjoberg 2004). In our work we could show that a small portion of the PRs are associated with Hsp90 in vivo in the cytoplasm, thus it is possible that Hsp90 can regulate cytoplasmic signaling pathway.

In contrast to the classically defined function of progesterone receptors as ligand-activated nuclear transcription factors, studies of amphibian oocyte maturation have suggested that PRs can mediate the activation of cytoplasmic signaling pathways. It is proposed that progesterone acts through amphibian plasma membrane PR to activate kinase pathways leading to MAPK (mitogen-activated protein kinase) activation (Lange 2004). It has been shown that p60-Src kinase and MAPK are activated by estradiol in MCF-7 cells and that p60-Src kinase interacts with PR and ER (Migliaccio et al. 1996). It has been also shown that progestin treatment of breast cancer cells causes a rapid and transient activation of MAPK signaling that is PR dependent (Migliaccio et al. 1998). MAPK activation by progestins can be blocked by antiprogestins and antiestrogens in COS-7 cells (Ballare et al. 2003). It has been suggested that MAPK is regulated by Hsp90 (Chiosis et al. 2004), but it is not known whether the interaction of the MAPK pathway and steroid receptors is regulated by Hsp90.

SUMMARY AND CONCLUSIONS

1. Steroid receptors exist as a large non-DNA-binding complex in hypotonic cell extracts that is composed of receptor, heat shock proteins and other proteins. There is, however, debate about the association in vivo of steroid receptors and Hsp in different compartments of target cells, especially in the nucleus. Due to the dynamic interaction between receptor-associated proteins and receptors, it is difficult to study the oligomeric complex in living cells. In our studies association of steroid receptors and Hsp90 in vivo was studied by using antibody αD, which can distinguish between the oligomeric and dissociated forms. We coexpressed cytoplasmic mutant PR with wt-Hsp90 and wt-PR with NLS-Hsp90 and used different means (cold, molybdate, in vivo crosslinking) to stabilize the complex in vivo in living cells. In vivo crosslinking was inefficient in demonstrating or inducing an oligomeric receptor form similar to that detected in vitro in cell homogenates. Molybdate and cold treatment were also used to stabilize the oligomeric complex. Treatments resulted in cytoplasmic accumulation of PR. αD predominantly stained nuclear PR, whereas the antibody that can recognize both receptor forms stains both the nuclear form and cytoplasmic form of the receptor. Analogously over-expressing wt-Hsp90 with the cytoplasmic form of PR decreased the αD staining but did not express nuclear form of Hsp90 with the wt-PR. These findings suggest that portion of the cytoplasmic receptor is seen as an oligomeric complex but in the nucleus most receptors were in dissociated form. The existence of the oligomeric complex has been supported by transfection studies in which Hsp90-NLS was co-expressed with a cytoplasmic mutant of the receptor. The nuclear and cytoplasmic receptor form was seen to co-translocate into the nucleus by the nuclear Hsp90. This was also seen in our experiments.

These results suggest that the major portion of the receptors in vivo are not associated with Hsp90 and that the oligomeric complexes seen in cell extracts may be formed during cell fractionation. This is further supported by the fact that nonliganded receptor monomers can be reconstituted to the oligomeric complex in various cell lysates (reticulocyte, mouse L cell, insect cell, plant cell) and homogenates. It may be suggested that the majority of PRs in the nucleus are associated with chromatin proteins

2. Nuclear proteins contain specific nuclear localization signals (NLSs) and nuclear export signals (NES) that are responsible for active nuclear targeting and export of proteins. Much less is known about the signals of the cytoplasmic location of the proteins apart from the protein location into mitochondria, peroxisomes or endoplasmic reticulum. Here we wanted to study the determinants of the cytoplasmic location of Hsp90. Sequences that dictate the nuclear localization of proteins were studied by constructing hybrid proteins between cytoplasmic proteins and potential nuclear localization signal containing polypeptide. Analogously we studied the sequences responsible for cytoplasmic localization by constructing hybrid molecules in which either the N-terminal or C-terminal of Hsp90 was fused with wild-type PR. The results showed that the N-terminal half does not interfere with the translocation, but that the C-terminal half prevents translocation into the nucleus. We also studied the strength of the cytoplasmic signal by constructing four chimeric molecules between C-terminal half of Hsp90 and ER with different numbers of proto-NLSs. The results suggest that Hsp90 contains a cytoplasmic localization sequence that does not function as NES but rather interacts with some cytoplasmic structures.

3. Hsp90 does not have NLS and it seems likely that the presence ofHsp90 in the nucleus is due to the fact that it is carried in by an NLS-containing client protein. Hsp90-dependent trafficking occurs along the cytoskeletal tract. The role of the Hsp90 in nuclear transport is not known. We studied the involvement of Hsp90 in the nuclear transport of PR by using digitonin permeabilized cells and

purified nuclei in in vitro transport systems. We demonstrated that unliganded PR, which was salt dissociated from Hsp90, was transported into the nucleus, whereas Hsp90 remained in the cytosol. When the oligomeric complex of the PR was stabilized with molybdate no nuclear accumulation was observed. When we added molybdate after ligand, PR accumulated in the nucleus. When the transport reaction was not supplemented with ATP, the nuclear transport of PR was inhibited. Our results suggest that Hsp90 assists the chaperoning process of PR preceding nuclear transport of the receptor and that the oligomeric complex is short-lived, dissociating before or during nuclear transport.

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