School of Medicine
Oregon Health Sciences University

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This is to certify that the Ph.D. thesis of

Mei-Shya Chen

has been approved

[redacted]
Professor in charge of thesis

[redacted]
Member

[redacted]
Member

[redacted]
Member

[redacted]
Member

[redacted]
Associate Dean for Graduate Studies

THE ROLE OF PHOSPHOPROTEIN PHOSPHATASE 5 IN GLUCOCORTICOID SIGNALING

by

Mei-Shya Chen

A DISSERTATION

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Table of Contents

Dedication

To my parents and family

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Abbreviations

ADP	Adenosine diphosphate
AMP-PNP	5'-adenylylimidodiphosphate
ANP	Atrial natriuretic peptide
AR	Androgen receptor
ATP	Adenosine triphosphate
ATPase	ATP hydrolase
BSA	Bovine serum albumin
CAT	Chloramphenicol acyltransferase
Cpr6	The yeast homologue of CyP-40
cpr6	The gene of Cpr6
Cpr7	The yeast homologue of CyP-40
cpr7	The gene of Cpr7
CyP-40	Cyclophilin 40 (a large immunophilin)
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetracetic acid, disodium salt
ER	Estrogen receptor
FCS	Fetal calf serum
FKBP51	FK506-binding protein (a large immunophilin)
FKBP52	FK506-binding protein (a large immunophilin)
FLAG epitope	DYKDDDDK
GR	Glucocorticoid receptor
HBD	Hormone binding domain
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hip	Hsp70-interacting protein
Hop	Hsp70/hsp90-organizing protein

hsp40	Heat shock protein 40
hsp70	Heat shock protein 70
hsp90	Heat shock protein 90
[^{3}H]TA	[^{3}H]triamcinolon acetonide
MR	Mineralocorticoid receptor
PBS	Phosphate-buffered saline
PDPK	Proline-directed protein kinase
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein phosphatase 1
PP1C	The catalytic subunit of PP1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B
PP2C	Protein phosphatase 2C
PP5	Protein phosphatase 5
PPIase	Peptidylproline *cis-trans* isomerase
PPT	The yeast homologue of PP5
PR	Progesterone receptor
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
TPR	Tetratricopeptide repeat

Abstract

The goal of this thesis is to address the biological function of phosphoprotein phosphatase 5 (PP5). The tetratricopeptide repeat (TPR) domain at the amino-terminus of PP5 is a targeting and regulatory domain. In an attempt to search for protein(s) associating with the TPR domain of PP5, extracts made from ^{35}S-labeled COS-7 cells overexpressing the FLAG-tagged TPR domain of PP5 were subjected to immunoprecipitation using an anti-FLAG antibody. A 90-kDa protein co-immunoprecipitated with the TPR domain of PP5. Since large immunophilins interact with hsp90 through their TPR domain, this 90-kDa protein was hypothesized to be hsp90. Immunoblotting using a monoclonal antibody to hsp90 confirmed this hypothesis. Hsp90 also co-immunoprecipitated with the FLAG-tagged full-length PP5 overexpressed in COS-7 cells, or with endogenous PP5 from untransfected COS-7 cells or rat brain. Thus, the interaction between the TPR domain of PP5 and hsp90 was not an artifact due to truncation or overexpression. Cellular fractionation and indirect immunofluorescence showed that the majority of PP5, like hsp90, is located in the cytoplasm. PP5 and hsp90 co-migrated in a peak of approximately 600-kDa during gel filtration, suggesting that PP5 and hsp90 may associate with other proteins to form a large complex. Since steroid receptors associate with hsp90, TPR proteins, and other proteins to form a large heterocomplex, I hypothesized that PP5 may be a component of a steroid receptor heterocomplex. PP5 co-immunoprecipitated with glucocorticoid receptor (GR) from L929 lysates, consistent with my hypothesis. In order to test the possible function of PP5 in GR heterocomplexes, the TPR domain of PP5 was used as a dominant negative mutant to test GR-mediated transcriptional activation. It inhibited GR-induced gene expression by approximately 65% - 85% in transfected CV-1 cells. Besides PP5, Hop and the large immunophilins, FKBP52 and CyP-40, are TPR proteins in GR heterocomplexes, and they interact with hsp90 through their TPR domains. It seemed possible that the inhibitory effect of the TPR domain of PP5 on GR-mediated transactivation was due to displacement of these TPR proteins, besides PP5, from GR

heterocomplexes. In order to test whether this might be possible, the TPR domains of Hop and FKBP 52 were used as controls to test their effects on GR-mediated transactivation. These TPR domains, unlike the TPR domain of PP5, did not significantly inhibit GR-mediated gene expression. This suggested that the inhibitory effect of the PP5 TPR domain on GR-mediated transactivation was due to specific displacement of PP5. The steroid receptor heterocomplex assembly pathway is dynamic and ordered. Besides hsp90, hsp70, Hop, and p23 co-immunoprecipitated with PP5, suggesting that PP5 is in both the intermediate heterocomplex containing Hop and the mature heterocomplex containing p23. Geldanamycin blocks the transition between intermediate and mature heterocomplexes. More hsp70 and Hop co-immunoprecipitated with PP5 in cells treated with geldanamycin than in control cells, and p23 did not co-immunoprecipitate with PP5 in the cells treated with geldanamycin. This further confirmed the presence of PP5 in the intermediate heterocomplex.

Introduction

Protein phosphorylation and dephosphorylation are universal processes for the posttranslational control of protein function. Protein kinases and phosphoprotein phosphatases mediate these processes. According to the residues phosphorylated or dephosphorylated, protein kinases and phosphoprotein phosphatases are divided into two families, respectively. They are the protein serine/threonine kinase family and protein tyrosine kinase family in the protein kinase superfamily, and the protein serine/threonine phosphatase family and protein tyrosine phosphatase family in the protein phosphatase superfamily. Based on sequence similarity, protein serine/threonine phosphatases are divided into two subfamilies which are designated PPP and PPM (Barford, 1996). The PPM subfamily is a large and varied protein phosphatase family present in both eukaryotes and prokaryotes. Protein phosphatase 2C (PP2C) is the defining member. The most abundant protein serine/threonine phosphatases of eukaryotes, protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), and protein phosphatase 2B (PP2B), belong to the PPP family. In general, the protein phosphatases in the PPP family have multiple functions (Barford, 1996). This is achieved by different regulatory or targeting subunits which bind to the same catalytic subunit of a protein phosphatase (Faux and Scott, 1996; Hubbard and Cohen, 1996). For example, PP2A is a heterocomplex that consists of a core heterodimer containing a PP2A catalytic subunit and an A-subunit, and a regulatory B-subunit. At least 15 distinct regulatory B-subunits have been found in PP2A. Moreover, protein phosphatase activity can be modulated by phosphorylation or dephosphorylation of regulatory subunits by protein kinases or phosphatases (Hubbard and Cohen, 1996). For example, the glycogen-targeting subunit of PP1, a regulatory subunit of PP1, can be phosphorylated by protein kinase A or insulin-stimulated protein kinase, and be dephosphorylated by PP2B, resulting in a change of substrate specificity.

I. Protein phosphatase 5

Protein phosphatase 5 (PP5) belongs to the PPP family. It was cloned by three laboratories in 1994. One used degenerate polymerase chain reaction (PCR) (Becker et al., 1994), and another screened a cDNA library at low stringency using a protein phosphatase 2B cDNA as a probe (Chen et al., 1994). In our laboratory, rat PP5 cDNA was cloned by the yeast two-hybrid system using the protein kinase-like domain of the atrial natriuretic peptide (ANP) receptor to search for interacting proteins (Chinkers, 1994). The ANP receptor is desensitized by serine/threonine dephosphorylation (Garbers and Lowe, 1994), so PP5 might be the phosphatase mediating the desensitization of the ANP receptor. The human PP5 gene is located on chromosome 19q13.3 (Yong et al., 1995). The molecular weight of PP5 is 58 kDa.

The phosphatase catalytic domain at the carboxyl-terminus of PP5 contains motifs typical for the PPP subfamily of protein serine/threonine phosphatases. When PP5 activity was characterized using ^{32}P-labeled phosphorylase as the substrate, PP5 was sensitive to the tumor promoter okadaic acid and microcystin, but not sensitive to inhibitors 1 and 2 (Chen et al., 1994). PP5 is less sensitive to okadaic acid than PP2A, but more sensitive than PP1. Like PP1 and PP2A, PP5 does not require divalent cations for activity, but its activity can be stimulated several fold by optimal concentrations of Mn^{2+} or Mg^{2+} (Chinkers and Wilson unpublished data) (Chen et al., 1994).

A tetratricopeptide repeat (TPR) is a degenerate 34-residue repeat, containing eight loosely conserved consensus residues. The secondary structure of a TPR consists of two α-helical domains and a proline-induced turn (Goebl and Yanagida, 1991; Lamb et al., 1995; Sikorski et al., 1990). Each domain contains both hydrophobic and hydrophilic faces. TPRs are frequently tandemly arrayed to form a TPR domain, although it is also common for single or double TPRs to be found separated from the arrays. TPR domains mediate protein-protein interactions. These interactions may involve intra- and/or intermolecular contacts, and TPR-TPR or TPR-non-TPR contacts. TPR proteins have been

discovered in a variety of subcellular locations, including nucleus, cytoplasm, and mitochondria. The cellular processes involving TPR proteins include cell-cycle control (Hirano et al., 1990; Lamb et al., 1994; Sikorski et al., 1990), transcriptional repression (Schultz et al., 1990; Tzamarias and Struhl, 1995), stress response (Honore et al., 1992; Nicolet and Craig, 1989), protein kinase inhibition (Barber et al., 1994; Lee et al., 1994), mitochondrial and peroxisomal protein transport (Brocard et al., 1994; Haucke et al., 1996), and RNA splicing (Legrain and Choulika, 1990).

PP5 contains four tandemly arranged TPRs at its amino-terminus. The first three begin in tandem, while two extra residues are located between the third and fourth motifs. The first two TPRs are the most similar to each other (Chen et al., 1994). The TPR domain of PP5 is most closely related to the TPR domains of the large immunophilins (FKBP52 and cyclophilin 40) and Hop (Chinkers, 1994). The part of PP5 interacting with the protein kinase-like domain of the ANP receptor is the TPR domain, suggesting that it is a targeting domain. It is not clear whether all four TPRs of PP5 are involved in this interaction. PP5 activity measured by using ^{32}P-labeled casein as substrate was stimulated more than 25 fold by proteolytic cleavage of the TPR domain (Chen and Cohen, 1997). This suggests that the TPR domain shields the active site of PP5, and functions as a regulatory domain as well as a targeting domain. Since protein phosphatases in the PPP subfamily have multiple functions, it seemed likely that the TPR domain of PP5 interacted with other proteins besides the protein kinase-like domain of the ANP receptor. This thesis will show that the TPR domain of PP5 interacts with heat shock protein 90 (hsp90).

The activity of purified bovine PP5 was stimulated 4-14 fold by arachidonic acid (Skinner et al., 1997). The half-maximal concentration for stimulation of PP5 by arachidonic acid was 50-100 μM. Oleic acid, linoleic acid, and palmitoleic acid also stimulated the PP5 activity. Saturated fatty acids and alcohol or methyl ester derivatives of fatty acids did not significantly affect the PP5 activity. These results suggested that PP5 might be regulated *in vivo* by a lipid second messenger. PPT is the yeast homologue of

PP5. The PPT null mutant is viable, and does not show increased sensitivity to high or low temperatures, high concentrations of salt, or to low pH (Chen et al., 1994). Although the biological functions of PP5 are not yet fully known, this thesis shows that PP5 may be important for steroid hormone receptor signaling.

II. Steroid receptors

1. Structure and function

Steroid hormones are synthesized and secreted by endocrine cells. These molecules, through binding to intracellular receptors, coordinate complex events involved in development, differentiation, and physiological responses to diverse stimuli. The steroid hormone receptors are transcription factors. Upon hormone binding, the hormone-receptor complexes activate the transcription of genes having cognate hormone response elements in their promoters. The classic steroid hormone receptor family consists of the receptors for glucocorticoids (GR), progesterones (PR), estrogens (ER), androgens (AR), and mineralocorticoids (MR). Amino acid sequence analysis and mutational dissection of receptors indicate that they can be subdivided into several domains (Tsai and O'Malley, 1994). The amino-terminal transactivation domain is highly variable in sequence and in length. This domain contains a transactivation function, which activates target genes, presumably by interacting with components of the core transcriptional machinery, coactivators, or other transactivators. Just downstream of the transactivation domain, the DNA-binding domain contains two type II zinc fingers which are responsible for DNA recognition and dimerization. The domain next to the DNA-binding domain is a hinge region which may allow the protein to bend or alter conformation, and often contains a nuclear localization domain (e.g. GR and PR) and/or transactivation domain (e.g. GR). The hormone-binding domain is located at the carboxyl-terminus, and is relatively large and functionally complex. It usually contains regions important for ligand binding, heat shock protein association, dimerization, nuclear localization, transactivation, and intracellular

repression (e.g. PR). In the absence of hormone, the hormone-binding regions can be regarded as repressors of receptor function, since if they are deleted, the truncated receptors become constitutive gene activators.

2. Cellular localization and receptor trafficking

Before steroid hormone binding, the steroid hormone receptor associates with several proteins to form heterocomplexes which exist either in the cytoplasm or nucleus (Smith and Toft, 1993). Upon binding their hormone ligands, the receptors undergo an activation (or transformation) step, and dissociate from complexes. Studies using heterokaryons have demonstrated that hormone-free PR, GR, and ER shuttle between the cytoplasm and the nucleus (DeFranco et al., 1995; Guiochon-Mantel et al., 1996; Guiochon-Mantel et al., 1991). This phenomenon is called receptor recycling. Steroid receptors are constantly passing into and out of the nuclei of hormone-free cells. Under steady state conditions most GR and MR molecules are detected in the cytoplasm, whereas most PR, ER, and AR molecules are in the nucleus. It is not clear what determines the steady state localization. In the presence of ligand, all steroid receptors are found in the nucleus. It has been proposed that steroid hormone receptors move along cytoskeletal networks to enter the nucleus (Pratt and Toft, 1997). After disruption of cytoskeleton by inhibitors (e.g. nocodazole, demelocin, or cytochalasin), however, hormones were still able to induce translocation of the Δ638-642 PR mutant into the nucleus (Guiochon-Mantel et al., 1996). The Δ638-642 PR mutant is missing its primary nuclear localization signal, and is cytoplasmic in the absence of hormone. This mutant retains biological activity. This observation suggests that the cytoskeleton is not involved in nuclear translocation.

3. Potential roles of receptor phosphorylation and dephosphorylation

The steroid receptors are phosphorylated in the unliganded form. They become hyperphosphorylated after association with hormones. A number of roles have been suggested for phosphorylation in the regulation of steroid receptor function. These include the regulation of transcriptional activity, receptor stability, and receptor nucleocytoplasmic

shuttling. Seven phosphorylated sites in the mouse GR have been identified by phosphopeptide mapping and sequencing (Bodwell et al., 1991). All of these sites are in the amino-terminal domain, and all are serines, except for threonine 159. Serines 122, 150, 212, 220, and 234, and the sequences surrounding them are conserved in the homologous regions of the rat and human GR. Serine 150 is also conserved in the human MR. The phosphorylation site at threonine 159 is conserved in the rat but not in the human GR. All but serine 315 are within the transactivation domain. These seven sites accounted for about 80% of the receptor associated ^{32}P (Ortí et al., 1992). Therefore most of the major sites have been identified, but other sites may still be uncovered. All the phosphorylated sites except serine 150 and 315 are in known kinase consensus sequences. Serine 122 is in a consensus sequence for casein kinase II. Threonine 159 and serines 212, 220, and 234 are in consensus sequences for proline-directed protein kinases (PDPK).

The role of phosphorylation in GR transactivation activity is controversial. Early studies suggested that mutation of single or multiple phosphorylated sites in mouse or human GR had little effect on the ability of these mutants to activate transcription (Almlöf et al., 1995; Mason and Housley, 1993). A recent study showed that single or multiple phosphorylation site mutations had little effect on mouse GR expression, subcellular distribution, or on its ability to activate hormone-mediated transcription from a complex promoter (derived from mouse mammary tumor virus). In contrast, the phosphorylation status of the mouse GR had a profound effect on its ability to transactivate a minimal promoter containing simple glucocorticoid response elements after hormone administration (Webster et al., 1997). Thus, the phosphorylation state of GR affects the transactivation activity of GR, which is dependent on the context of promoters. Ligand-dependent down-regulation by glucocorticoids of both receptor mRNA and protein was abrogated in mutants containing three or more phosphorylation site alterations. Thus, receptor phosphorylation may play a crucial role in regulating receptor levels and hence control receptor function. Moreover, promoter complexity and context may affect the ability of various

phosphorylated forms of the GR to regulate transcription. The hyperphosphorylation of GR is induced by glucocorticoid agonists. The magnitude of the increase in phosphate ranged from 70% to 3 to 4-fold, and was mostly due to phosphorylation of the amino-terminal domain. In contrast, the hyperphosphorylation of GR is not induced by the antagonist RU486 which is ineffective in causing GR activation and nuclear translocation (Ortí et al., 1989b). This suggests that activation and perhaps nuclear localization are required for hyperphosphorylation.

4. GR recycling affected by phosphorylation and dephosphorylation

The kinetics of GR phosphorylation in intact cells showed that hormone-induced phosphorylation was seen as early as 5 min after adding hormone and persisted for 20 h. Analysis of newly formed cytosolic and nuclear-bound activated GRs showed that activation precedes hyperphosphorylation. The rate of receptor dephosphorylation was much slower than the rate of phosphorylation or of hormone dissociation and appeared to be slightly increased by agonist. Thus, most hyperphosphorylated GRs are recycled and reutilized in hyperphosphorylated form (Bodwell et al., 1993; Ortí et al., 1993). In the absence of hormone, GR recycled slowly via a path that involved dephosphorylation, whereas in the presence of hormone, GRs recycled through two pathways, a major one, initiated by hormone dissociation but not accompanied by dephosphorylation, and a quantitatively minor one that involves dephosphorylation (Ortí et al., 1992). GRs may be reutilized following dissociation of hormone and release from the nucleus through a process which presumably requires hsp90 (Ortí et al., 1989b). Nuclear import or export of hormone agonist-bound GRs was not affected by okadaic acid, whereas 100 nM okadaic acid led to inefficient nuclear retention of agonist-bound GRs, and their redistribution into cytoplasm. These receptors appeared to be trapped in the cytoplasmic compartment and were unable to re-enter the nucleus. Adding okadaic acid during different steps of GR recycling demonstrated that okadaic acid must be present during nuclear export of GRs to block GR recycling (DeFranco et al., 1991). This suggested that a protein phosphatase(s)

was involved in nuclear re-entry of GR. Indirect immunofluorescence analyses demonstrated that hormone insensitivity in v-mos transformed cells was associated with inefficient nuclear retention of GR. Desensitized receptors appeared to retain some capacity to bind hormone *in vivo*. Thus alterations in the intracellular partitioning of GR in v-mos transfected cells resulted in the generation of a novel desensitized receptor that was apparently trapped in the cytoplasm and incapable of being reutilized (Qi et al., 1989). This similarity between the v-mos transformed cells and the cells treated with okadaic acid suggested that v-mos might directly or indirectly act on the protein phosphatase. Treatment of v-mos transformed cells with okadaic acid led to the reversal of oncoprotein effects on GR recycling and retention of receptors within the nuclear compartment. Thus, the phosphorylation state of GR might affect GR recycling, and it was proposed that GR recycling was influenced by the activity of a protein phosphatase and that the influence of this pathway observed in v-mos transformed cells may be the result of effects of the oncoprotein on the phosphatase or a specific subset of its targets (DeFranco et al., 1991).

5. GR phosphorylation state and activity during the cell cycle

In the absence of hormone, cytoplasmic GRs are present throughout the cell cycle. More GR is present during the S and G2 phases than during the other cell cycle phases. After hormone treatment, high levels of nuclear GR are maintained in late G1 and S phases, and lower numbers are found in G2, M, and early G1. To be effective, glucocorticoids must be present in the late G1 and S. Glucocorticoids are ineffective in G2, M, and early G1 (Cidlowski and Cidlowski, 1982). GRs that translocated to the nucleus of G2 synchronized cells in response to dexamethasone treatment were not efficiently retained there and redistributed to the cytoplasmic compartment. In synchronized cells, the GR was transcriptionally inactive in the G2 phase of the cell cycle, and its phosphorylation pattern was altered relative to that in asynchronous cells, suggesting that specific protein kinases and phosphatases regulated by cell cycle could affect nuclear retention and recycling of the GR (Hsu et al., 1992).

6. Steroid receptor heterocomplex assembly in an *in vitro* reconstitution system

Before activation, steroid receptors exist in heterocomplexes containing several proteins (Pratt and Toft, 1997). Because large amounts of PR can be immunoadsorbed from the oviducts of estrogen-treated chickens, this system has been used for purification of receptor heterocomplexes. Hip, heat shock protein 70 (hsp70), Hop, heat shock protein 90 (hsp90), p23, and large immunophilins (FK506-binding immunophilins FKBP51 and FKBP52, and cyclosporin A-binding immunophilin CyP-40), which are involved in the early, intermediate, or mature receptor heterocomplexes (Fig. 1), were identified in receptor immune pellets using this system (Milad et al., 1995; Prapapanich et al., 1996a; Smith et al., 1990). In order to understand the assembly pathway of steroid receptor heterocomplexes, PR and GR heterocomplex formation were studied using an *in vitro* reconstitution system. The receptor heterocomplexes can be assembled under cell-free conditions by incubating immunoadsorbed, hormone-free steroid receptors, which were preincubated with salt to strip them free of associated proteins, with rabbit reticulocyte lysate or concentrated lysate of steroid receptor-free cells (Dittmar et al., 1996; Scherrer et al., 1990; Stancato et al., 1996a). The receptor heterocomplexes assembled in reticulocyte lysate, like native receptor heterocomplexes, have steroid-binding activity, and show the hormone and temperature-dependent conversion of the 9S form (receptor heterocomplexes) to the 4S form (receptors alone). Several factors required for steroid receptor heterocomplex assembly by reticulocyte lysate have been defined. Heterocomplex assembly is an ATP/Mg^{2+}-dependent process that requires the presence of a monovalent cation, such as K^+ or NH_4^+ (Hutchison et al., 1992; Smith et al., 1992). In a careful study of the time course of heterocomplex assembly in reticulocyte lysate, Hip, Hop and hsp70 were recovered with the steroid receptor immune pellet immediately, with peak binding observed at about 4 min and recovery of these proteins decreasing as the incubation was continued at 30°C (Smith, 1993). In contrast, the recovery of p23 was not evident until 2 min, and it

reach a plateau from which there was no decline. Hsp90 binding attained a half-maximal level between 2-3 min of incubation and achieved a maximal level between 5-10 min. Hsp90 binding remained constant over the next 50 min. This information became the basis for a model of steroid receptor heterocomplex assembly.

7. Studies on the receptor heterocomplex assembly pathway using molybdate, AMP-PNP, geldanamycin, and immunodepletion

Molybdate, 5'-adenylylimidodiphosphate (AMP-PNP; a nonhydrolyzable ATP analog), and geldanamycin (a natural antitumor antibiotic that blocks ATP binding to hsp90) have been used to address the assembly pathway of steroid receptor heterocomplex. Molybdate stabilizes the mature steroid receptor heterocomplex containing steroid receptor, hsp90, p23, and one of the large immunophilins (Johnson and Toft, 1995). These receptor-hsp90-p23-large immunophilin complexes are dynamic without molybdate treatment (Smith, 1993; Smith et al., 1995). It seems likely that the site of molybdate interaction with the heterocomplex lies in hsp90. It has been reported that hsp90 binds ATP (Prodromou et al., 1997), and circular dichroism analysis indicates that ATP induced an increase in ß-pleated sheet content of hsp90 (Csermely et al., 1993). Molybdate induces a similar change in the conformation of hsp90. Thus, it may interact with the ATP-binding site of hsp90, stabilizing a conformation of hsp90 with increased affinity for the steroid receptor. Treatment of intact cells with molybdate trapped both GR and PR in the cytoplasm after steroid treatment (Yang and DeFranco, 1996). This effect of molybdate on GR and PR nuclear import *in vivo* is caused by an inhibition of the steroid receptor-hsp90-p23-large immunophilin complex dissociation after hormone treatment.

In the reticulocyte lysate reconstitution system, AMP-PNP treatment did not support the formation of steroid receptor-hsp90-p23-large immunophilin complexes, indicating that ATP hydrolysis is necessary for the formation of this mature steroid receptor heterocomplex (Johnson and Toft, 1995; Smith et al., 1992). Geldanamycin affected the formation of receptor heterocomplexes in reticulocyte lysate and in intact cells by increasing

recovery of the intermediate steroid receptor heterocomplexes containing receptor, hsp90, hsp70 and Hop, and eliminating the mature receptor heterocomplex containing receptor, hsp90, p23, and one of the large immunophilins (Johnson and Toft, 1995; Smith et al., 1995). Moreover, it was shown that geldanamycin treatment of intact cells caused the steroid receptor to lose steroid binding activity (Smith et al., 1995), inhibited the dexamethasone-dependent transactivating activity of GR (Whitesell and Cook, 1996), and impeded dexamethasone-dependent trafficking of the GR from the cytoplasm to the nucleus (Czar et al., 1997). Crystal structure studies show that geldanamycin binds to the ADP/ATP site of hsp90 (Prodromou et al., 1997; Stebbins et al., 1997), suggesting that geldanamycin acts by blocking the binding of nucleotides to hsp90. Thus, geldanamycin binding to hsp90 blocks mature receptor heterocomplex assembly but allows the accumulation of a Hop-containing intermediate state (Fig. 2).

Immunodepletion experiments also provide some information about the assembly pathway of steroid receptor heterocomplex. Immunodepletion of p23 from reticulocyte lysate prevented the formation of mature steroid receptor heterocomplex in this reconstitution system (Johnson and Toft, 1994), indicating p23 was required for the formation of stable mature steroid receptor heterocomplex. Addition of a monoclonal antibody against Hop to the reticulocyte lysate reconstitution system could inhibit the formation of mature receptor heterocomplex, but enhance the recovery of early steroid receptor heterocomplexes containing hsp70 and Hip. Thus, Hop functioned at the intermediate stage of steroid receptor heterocomplex assembly to facilitate the formation of subsequent steroid receptor heterocomplexes (Chen et al., 1996).

8. A model for the steroid receptor heterocomplex assembly pathway

Based on the reported information, a model for the assembly pathway of steroid receptor heterocomplex was proposed (Fig. 1). In this model, the steroid receptor heterocomplex assembly is a dynamic and ordered process. The steroid receptor-hsp70-Hip complex (an early heterocomplex) ---> the steroid receptor-hsp70-Hip-Hop-hsp90 complex

(an intermediate heterocomplex) ---> the steroid receptor-hsp90-p23-large immunophilin (a mature heterocomplex) (Frydman and Hohfeld, 1997; Smith et al., 1995). In reticulocyte lysate and in intact cells, receptor heterocomplex assembly is occurring simultaneously with disassembly (Scherrer et al., 1990; Smith, 1993). The formation of mature heterocomplexes requires ATP hydrolysis. Geldanamycin blocks the transition from an intermediate heterocomplex to a mature heterocomplex. In the steroid receptor heterocomplex, hsp90 is dimeric (Bresnick et al., 1990; Rexin et al., 1988). The steroid receptor in the mature heterocomplex has high affinity for hormone binding and is biologically active.

9. The proteins required to refold the steroid receptor to the steroid-binding state are preassociated with each other, and function as a self-sufficient protein folding machine

Rabbit reticulocyte lysate does not contain steroid receptors. When hsp90 was immunoadsorbed from reticulocyte lysate with a monoclonal antibody, the washed immunopellet contained all the factors required for receptor heterocomplex assembly and activation of steroid-binding activity (Hutchison et al., 1994b). Thus, the components of reticulocyte lysate required to refold the GR to the steroid binding state are preassociated with each other and act as a self-sufficient protein folding machine.

The immune complexes, containing hsp90, hsp70, and Hop, isolated from reticulocyte lysate are able to renature thermally denatured firefly luciferase up to 35% in an ATP-dependent manner (Schumacher et al., 1994). Moreover, when the GR was incubated with purified rabbit hsp90 and hsp70, and bacterial lysate containing human Hop, in the presence of an ATP-regenerating system and [^{3}H]triamcinolon acetonide ([^{3}H]TA; a GR ligand), the GR could bind to [^{3}H]TA. If the GR was incubated with these factors, but without [^{3}H]TA, the washed GR could not bind to [^{3}H]TA. Whereas, if molybdate or p23 was also present during incubation, the steroid-binding activity could be detected by incubating the washed GR with [^{3}H]TA. These results suggested that hsp90, hsp70, and

Hop worked together as a chaperone complex that possessed all of the folding/unfolding activity necessary to promote the conformation of GR with high affinity for steroid binding (Dittmar and Pratt, 1997). Moreover, this receptor-hsp70-Hop-hsp90 complex is not stable. Immunoadsorption of p23 from reticulocyte lysate yielded a complex containing p23, hsp90, and large immunophilins (Johnson and Toft, 1994). The complex forms rapidly in the presence of ATP, magnesium, and elevated temperature. The hsp70 appears to be only a minor component in p23 complexes, but it may be an active participant in the assembly of these complexes. If reticulocyte lysate was treated with molybdate, a high level of Hop was present in the p23 complex (Johnson and Toft, 1995). A lower level of Hop was present in the p23 complex in the absence of molybdate. The ordered assembly model does not account for why these proteins, required for the assembly of steroid receptor into a functional heterocomplex, are preassociated in a self-sufficient protein-folding structure. Thus, the mechanism for the assembly of steroid receptor heterocomplexes needs further study.

10. Receptor transformation

Steroid receptor transformation measured by 9S (receptor heterocomplexes) to 4S (receptors alone) conversion or by acquisition of DNA-binding activity is both hormone-dependent and temperature-dependent (Denis et al., 1988; Schmidt et al., 1985). In cytosol preparations, steroids do not promote GR or PR transformation at 0°C, but do so at room temperature. Since steroid binding occurs at both 0°C and 25°C, the temperature-dependent step must occur after steroid binding. Based purely on observations in cytosol or purified systems, a model of receptor transformation was proposed (Stancato et al., 1996b; Tsai and O'Malley, 1994). In the absence of ligand, the receptor exists as a large heterocomplex containing hsp90 and several proteins, which has steroid hormone binding activity. In this complex, the receptor exists in a conformation such that the dimerization domain is not available and the transactivation domain is under the control of the intramolecular repressor. Hsp90 is conceived as trapping the receptor hormone-binding domain (HBD) in a partially

unfolded conformation. After binding of steroid in the steroid-binding pocket, temperature promotes the continued folding of the HBD, and then the dissociation of hsp90 from the receptor HBD, which results in unmasking the DNA-binding domain, receptor dimerization sites, transactivation domain, and in the case of GR and MR, nuclear localization signals as well. The hormone-binding pocket becomes buried and is no longer accessible to steroid. The steroid receptors dissociate from the receptor heterocomplexes, become dimeric, bind to the hormone response elements, and activate the transcription of the target gene.

11. Alkaline phosphatase treatment promotes GR activation

The addition of calf intestinal alkaline phosphatase stimulates the rate of activation of [^{3}H]triamcinolon acetonide-GR complexes in rat liver cytosol (0°C, 2 h). The extent of activation has been quantitated by measuring the binding of [^{3}H]triamcinolon acetonide-GR complexes to DNA-cellulose (Barnett et al., 1980; Gruol and Wolf, 1989). Moreover, addition of bovine intestinal alkaline phosphatase to mouse AtT-20 cell cytosol or rat liver cytosol increases the rate of GR transformation, as evidenced by a change in sedimentation rate from 9.1S to 5.2S (Matic and Trajkovic, 1986). Acid phosphatases are completely ineffective. Alkaline phosphatase-promoted GR transformation is both time and dose-dependent (Reker et al., 1987). This suggests that dephosphorylation of some component in the cytosol is involved in the destabilization of GR heterocomplexes, resulting in GR transformation.

12. Summary

Steroid hormone receptors are transcription factors. Before steroid hormone binding, the steroid hormone receptors associate with several proteins to form heterocomplexes which are either in the cytoplasm or nucleus. Upon binding their hormone ligands, receptors undergo an activation (or transformation) step, dissociate from complexes, can bind to the cognate hormone response elements, and activate the transcription of target genes. Steroid receptors are phosphorylated in the unliganded from. They become hyperphosphorylated after association with hormone. Site-directed mutagenesis studies

showed that the phosphorylation state of GR had a profound effect on its ability to transactivate a minimal promoter, but not a complex promoter. GR recycling was affected in cells treated with okadaic acid or/and in v-mos transformed cells. This suggested that protein phosphatase(s) and v-mos (a protein kinase) influence GR recycling, and that the phosphorylation state of GR may affect GR recycling. Alkaline phosphatase stimulates the activation of GR complexes in cytosol. This suggests that dephosphorylation of some components in the cytosol results in GR transformation. Although the mechanisms for the GR phosphorylation state affecting GR functions are unclear and complicated, protein phosphatases and protein kinases are involved in these processes.

The steroid receptor heterocomplex assembly pathway (Fig. 1) is a dynamic and ordered process. The mature steroid receptor heterocomplex has high affinity for hormone binding and is biologically active. The formation of mature heterocomplexes requires ATP hydrolysis.

III. Hsp90

1. The general properties of hsp90

In the absence of ligand, the steroid receptors associate with different proteins at different stages in the heterocomplex assembly pathway. The proteins associating with steroid receptors include hsp90, hsp70, hsp40, Hip, Hop, p23, and large immunophilins. The roles of most members of the heterocomplexes are unclear. Some *in vitro* experiments suggest that some of these proteins play a role in establishing the heterocomplexes. The role of hsp90 in the steroid heterocomplex is the most studied. Hsp90 is the most abundant constitutive heat shock protein in eukaryotic cells, accounting for 1-2% of cytosolic protein. Eukaryotic hsp90 has two isoforms, which are encoded by separate genes. These two isoforms are called hsp90α and hsp90ß in human cells (Hickey et al., 1989), hsp86 and hsp84 in murine cells (Moore et al., 1989; Perdew et al., 1993), or hsp82 and hsc82 in yeast (Picard et al., 1990). "Hsp90" is used to represent these two isoforms in all species

in this thesis. Hsp90 is present mainly as homodimers in cells (Perdew et al., 1993). Hsp90 dimerization is required for function *in vivo* (Minami et al., 1994). The carboxyl-terminal 49 amino acids from human hsp90 are required for both dimer formation and rescue of a lethal double hsp90 mutant of haploid yeast (Minami et al., 1994). The exact function(s) of hsp90 in the cell has not been defined. It has been clearly shown that hsp90 functions as a molecular chaperone, facilitating the folding of proteins *in vitro* (Freeman et al., 1996; Wiech et al., 1992). The crystal structure study shows that hsp90 has an ADP/ATP-binding site at its amino-terminus (Prodromou et al., 1997). The ATP affinity of hsp90 (about 132 μM) is sufficiently high to guarantee that the ATP-binding site will be fully occupied at cellular ATP concentrations. Conversely, the affinity for ADP (29 μM), although higher than for ATP, is probably insufficient to saturate the binding site with ADP at cellular concentrations. According to the affinity of hsp90 for ATP and ADP, hsp90 goes through an ATP-binding --> ATP-hydrolysis --> ADP-release cycle. Whether or not hsp90 has ATPase activity is somewhat controversial. The crystal structural data suggest that it is highly possible that hsp90 has ATPase activity (Prodromou et al., 1997). Moreover, purified hsp90s have been reported to have ATPase activity (Nadeau et al., 1993). Hsp90 is heavily phosphorylated, and its phosphorylation state seems to affect its function. Purified hsp90 can stimulate the heme-sensitive eIF-2α kinase activity. This stimulatory effect is abolished after incubation of hsp90 with a highly purified type 1 phosphoprotein phosphatase (Szyszka et al., 1989). It has been reported that the free, GR-associated, and dissociated forms of hsp90 have the same phosphate content, indicating that net changes in hsp90 phosphorylation are unlikely to be involved in regulation of GR function in the intact cell (Orti et al., 1989a). Whereas, the possibility of changes in phosphorylation and dephosphorylation of some sites cannot be excluded. The localization of hsp90 is predominantly in the cytoplasm (Gasc et al., 1990; Lai et al., 1984), but some hsp90 is also present in the nucleus. It has been reported that some hsp90 localizes to the cytoskeleton (Czar et al., 1996; Fostinis et al., 1992), and proposed that cytoskeleton-

associated hsp90 may reflect potential protein targeting and trafficking functions of hsp90. In addition to association with steroid receptors, hsp90 associates with a variety of proteins including transcription factors, protein kinases, cytoskeleton, and proteasome (Pratt and Toft, 1997).

2. The interaction between hsp90 and the components of a steroid receptor heterocomplex, and the role of hsp90 in steroid receptor signaling

Hsp90 is dimeric in a steroid receptor heterocomplex (Bresnick et al., 1990; Rexin et al., 1988). Deletion mutagenesis showed that hsp90 interacts with the hormone-binding domain (HBD) of steroid receptor (Scherrer et al., 1993; Schowalter et al., 1991). The HBD of GR must be bound to hsp90 to be in a high-affinity steroid binding conformation (Bresnick et al., 1989; Scherrer et al., 1990). Some *in vitro* experiments have also suggested that hsp90 has a negative role, keeping the DNA binding activity of the receptor repressed (Nemoto et al., 1990; Sanchez et al., 1987; Scherrer et al., 1990). Besides the site for interacting with the steroid receptor, hsp90 has sites interacting with Hop, p23, and large immunophilins. It is not clear which parts of hsp90 are involved in these interactions.

The GR, MR, and ER have altered transcriptional activating activity in mutant yeast cells expressing about 5% of the normal amount of hsp90 (Picard et al., 1990). At this low hsp90 level, the signaling pathways are functional but require much higher concentrations of steroid than at the normal hsp90 level. Moreover, a truncated constitutive GR lacking the HBD was equally active at low and normal hsp90 levels, showing that transcriptional activation *per se* was not affected (Picard et al., 1990). This suggests that hsp90 is critical for signal transduction by steroids *in vivo*, and the hsp90-receptor interaction would have two consequences: it would establish or stabilize the conformation of the active hormone-binding domain, and it would inactivate other functional domains in a manner that would readily be reversed upon hormone binding and hsp90 release. Furthermore, point mutations in hsp90 also affect the steroid-signaling pathway in yeast (Bohen, 1995; Bohen and Yamamoto, 1993; Nathan and Lindquist, 1995). These observations also suggested

that hsp90 is important for steroid receptor signaling. There is a unique natural demonstration of the *in vivo* requirement of hsp90 for steroid binding (Kaufmann et al., 1992). Hsp90 present in the early germ cells of rodents is absent from the epididymal sperm, which contain full-length GR but do not have glucocorticoid-binding activity.

IV. Hsp70

1. The general properties of hsp70

There are two forms of higher eukaryotic hsp70 existing in the cytoplasm, a 73-kDa form that is constitutively expressed (hsc70), and a 72-kDa stress-inducible form (hsp70) (Hartl, 1996). Although the dominant form associated with the steroid receptor is hsc70, "hsp70" was used in this thesis to represent hsc70. A cycle of hsp70 interacting with and dissociating from the chaperoned protein has been proposed (Palleros et al., 1993). The ADP-bound form of hsp70 has a high binding affinity for unfolded substrates. A cofactor, such as GrpE and its homologues, functions as a nucleotide-exchange protein, promoting the ADP-bound form hsp70 to become the ATP-bound form. This ATP-bound form of hsp70 still interacts with the chaperoned protein, but with lower affinity. A physiological concentration of K^+ facilitates the dissociation of the chaperoned protein from the ATP-bound form of hsp70. Subsequently, the ATP bound to hsp70 is hydrolyzed and hsp70 becomes the ADP-bound form again. The ATP-induced dissociation of hsp70 from the protein being chaperoned requires K^+. It is thought that the monovalent cation is required for receptor heterocomplex assembly *in vitro*, which is related to the role of hsp70 in this process.

2. The role of hsp70 in steroid receptor signaling

The site of receptor interacting with hsp70 is the HBD (Schowalter et al., 1991). The sites for hsp70 and hsp90 in the receptor could not be separated, and it is thought that they both interact with the HBD through interactions at multiple locations or through some

structural quality (hydrophobicity) that is distributed through out the HBD (Schowalter et al., 1991). Addition of a monoclonal anti-hsp70 antibody to the reticulocyte lysate inhibited the receptor heterocomplex assembly (Smith et al., 1992). Furthermore, reticulocyte lysate depleted of hsp70 by adsorption with ATP-agarose, which binds hsp70 with high affinity, blocked receptor heterocomplex formation, but it could be reactivated by adding purified hsp70 (Hutchison et al., 1994a). Thus, hsp70 is required for receptor heterocomplex assembly. Purified hsp70 alone can bind to the GR, but there is no steroid-binding activity unless hsp90 and the other components of the heterocomplex assembly system are present (Hutchison et al., 1994a).

V. Hsp70 co-chaperones - hsp40, Hip, and Hop

There are three proteins in the receptor heterocomplex, assisting the chaperone activity of hsp70. They are hsp40, Hip, and Hop. Hsp40 stimulates the ATPase activity of hsp70 (Hohfeld et al., 1995). Mutations in Ydj1, a yeast hsp40 homologue, affect steroid receptor function in yeast (Caplan et al., 1995; Kimura et al., 1995). Thus, it is proposed that hsp40 is involved at some point in the receptor-chaperoning process. However, hsp40 has not been found in the heterocomplex.

Hip binds to the ATPase domain of hsp70, stabilizing the ADP state of hsp70 that has a high affinity for substrate protein (Höhfeld et al., 1995). Hip interacts with hsp70 through its TPR domain (Prapapanich et al., 1996b). It appears to be ubiquitously expressed, and when it is immunoadsorbed from reticulocyte lysate, substantial amounts of hsp70 and smaller amount of hsp90 and Hop are co-adsorbed (Prapapanich et al., 1996a). Hip is recovered at the early times of receptor heterocomplex assembly in reticulocyte lysate (Smith, 1993). A requirement for Hip in the receptor heterocomplex assembly has not yet been demonstrated.

Hop contains 6-8 TPRs (Honoré et al., 1992). The yeast Hop homologue is called Sti1 (Nicolet and Craig, 1989). Yeast carrying a disruption of the Sti1 gene grew normally at

30°C but showed impaired growth at higher and lower temperatures. Mutational analysis of murine Hop indicates that Hop binds independently to hsp70 via an amino-terminal TPR domain and to hsp90 via a central TPR domain (Chen et al., 1996; Lässle et al., 1997). Hop has an ADP/ATP exchange activity for hsp70 (Gross and Hessefort, 1996). The receptor heterocomplex containing Hop is in the transition state of the assembly pathway (Chen et al., 1996). In a reconstituted reticulocyte lysate system, immune depletion of Hop inactivates heterocomplex assembly, and assembly activity is reactivated by the addition of bacterially expressed Human Hop (Dittmar et al., 1996). Thus, Hop is required for receptor heterocomplex assembly. Deletion of Sti1 reduced GR-mediated transactivation activity in yeast cells (Chang et al., 1997), suggesting that Hop plays a role in steroid receptor signaling *in vivo*.

VI. p23

P23 is a component in the mature steroid receptor heterocomplex, and has chaperone activity *in vitro* (Bose et al., 1996; Freeman et al., 1996). Purified p23 can bind to purified hsp90, which requires Mg^{2+}-ATP, and molybdate enhances this interaction (Johnson et al., 1996). This explains, in part, the known effects of ATP and molybdate on the assembly of mature receptor heterocomplex. The mature receptor heterocomplex contains p23, and its formation requires Mg^{2+}-ATP hydrolysis (Johnson and Toft, 1995), but is blocked by geldanamycin. P23 is a phosphorylated protein (Johnson et al., 1994), but it has not yet been determined whether the phosphorylation state of p23 is important for binding to hsp90 or for the formation of mature receptor heterocomplex. Immunodepletion of p23 from reticulocyte lysate inactivates the mature receptor heterocomplex assembly, and assembly is reactivated by the addition of purified p23 (Hutchison et al., 1995; Johnson and Toft, 1994). Thus, p23 action is that its binding to hsp90 stabilizes the mature receptor heterocomplex formation.

VII. Large immunophilins - FKBP51, FKBP52, and CyP-40

1. The general properties of large immunophilins

The mature steroid receptor heterocomplexes contain large immunophilins. All members of the immunophilin family possess peptidylproline *cis -trans* -isomerase (PPIase) activities, suggesting that they may play a role in protein folding in the cell (Galat, 1993). Immunophilins are divided into two classes. The FKBPs are binding proteins for the immunosuppressant drugs, FK506 and rapamycin, while the cyclophilins (CyPs) bind to the immunosuppressant drug, cyclosporin A (Galat, 1993). Three higher molecular weight immunophilins, FKBP51, FKBP52, and cyclophilin 40 (CyP-40), have been discovered as components of the steroid receptor complexes (Milad et al., 1995; Ratajczak et al., 1993; Smith et al., 1993a; Smith et al., 1993b; Tai et al., 1992). FKBP52 and CyP-40 have chaperone activities *in vitro* (Bose et al., 1996; Freeman et al., 1996). Each of these three large immunophilins possesses three TPRs and a calmodulin binding domain in their carboxyl-terminal half (Callebaut et al., 1992; Massol et al., 1992; Nair et al., 1997; Ratajczak et al., 1993). Their amino-terminus contains a PPIase catalytic domain. Deletion mutagenesis showed that FKBP52 and CyP-40 interact with hsp90 through their TPR domains plus short flanking regions of acidic and basic residues clustered at the amino- and carboxyl-terminal ends of the TPR domain (Radanyi et al., 1994; Ratajczak and Carrello, 1996).

2. The mature steroid receptor heterocomplex contains one of the large immunophilins

Immunoadsorption of cytosol with anti-FKBP52 antibody or passage of cytosol through an FK506 affinity matrix yielded co-retention of hsp90 but not CyP-40, and immunoadsorption with anti-CyP-40 or passage through a cyclosporin A affinity column yielded co-retention of hsp90 but not FKBP52 (Owens-Grillo et al., 1996; Renoir et al., 1995). Furthermore, both purified CyP-40 and FKBP52 bound directly to purified hsp90, and excess CyP-40 blocked the binding of FKBP52 (Owens-Grillo et al., 1995). These

observations all support the conclusion that there is a common immunophilin-binding region on hsp90 and that immunophilins exist in separate complexes with hsp90. This thesis will show that there is also a subset of these complexes containing PP5 in place of a large immunophilin.

3. The possible role of large immunophilin in steroid receptor signaling

The function of the immunophilins in steroid receptor heterocomplexes has not been defined. The immunophilins have been proposed to play a role in receptor heterocomplex assembly. However, GR heterocomplexes with steroid binding ability have been assembled with purified hsp90, purified hsp70, purified p23, and bacterial lysate containing human Hop (Dittmar et al., 1996). Indirect immunofluorescence showed that the majority of FKBP52 was located in the nucleus, except for the nucleoli, with substantial amount in the cytoplasm (Czar et al., 1994; Perrot-Applanat et al., 1995; Ruff et al., 1992). The cytoplasmic FKBP52 colocalized with cytoskeleton. Because the majority of FKBP52 was in the nucleus and the majority of cytoplasmic FKBP52 was colocalized with cytoskeleton, it was proposed that FKBP52 may play a role in receptor trafficking from the cytoplasm to the nucleus. Injection of an antibody against the negatively charged peptide EDLTDDED, amino acids 140-147 of rabbit FKBP52 (containing six negatively charged residues), into L929 cells impeded subsequent dexamethasone-mediated nuclear translocation of the GR (Czar et al., 1995). Nonimmune serum, an antibody raised against another site on FKBP52, and an antibody against this negatively charged sequence blocked by preincubation with purified FKBP52 did not affect receptor trafficking. Thus, FKBP52 may play a role in the translocation of the GR and may serve as a nuclear localization signal-binding protein interacting with the nuclear localization signal 1 of GR. Two acidic residues in this negatively charged region implicated in the interaction are absent in the FKBP51 sequence (Baughman et al., 1995). Thus, FKBP51 would not be expected to function as a nuclear localization signal-binding protein like FKBP52. The molecular components that mediate steroid receptor signal transduction in eukaryotes are highly

conserved, and mammalian GR exhibits faithful hormone-dependent transcriptional activity in yeast. Cpr6 and Cpr7 are the only CyP-40 homologues in *Saccharomyces cerevisiae*. The yeast *cpr7* (the gene of Cpr7) deleted mutant expressed about one-fifth of the hormone-mediated transactivation activity of GR detected in wild-type cells. Deletion of *cpr6* (the gene of Cpr6) from wild-type cells did not result in decreased hormone-dependent activity of GR, nor did it further decrease GR activity in *cpr7*Δ cells. Deletion of *cpr7* had only a small effect on the activity of a truncated form of GR that activates reporter gene expression in a constitutive and hsp90-independent manner. Thus, the role of Cpr7 in steroid receptor signal transduction appears to be in events that are regulated by hsp90 (Duina et al., 1996).

VIII. Summary

Steroid receptor heterocomplex assembly is dynamic. A model for the assembly pathway of steroid receptor is: a receptor-hsp70-Hip complex --> a receptor-hsp70-Hip-Hop-hsp90 complex --> a hsp90-p23-large immunophilin complex. The roles of some of these proteins in the receptor assembly have been defined. This thesis will show that PP5 interacts with hsp90 through its TPR domain, and is a component of the steroid receptor heterocomplex. A PP5 deletion mutant containing the TPR domain of PP5, without the catalytic domain, can inhibit GR-mediated gene expression, suggesting that PP5 may be important for GR signaling. Some results of this thesis have been published in The Journal of Biological Chemistry (Chen et al., 1996).

Materials and Methods

Tissue Culture

COS-7, CV-1, and HeLa cells were cultured in DMEM containing 10% fetal calf serum (FCS). L929 mouse fibroblasts (L cells) were grown in DMEM containing 10% FCS or 10% iron-supplemented calf serum. Sf9 cells were grown in suspension culture in Grace's medium supplemented with lactalbumin hydrolysate, Yeastolate (Life Technologies, Inc.), and 10% FCS.

Construction of plasmids

Plasmids expressing FLAG-tagged PP5 (pCMV6-FLAG-PP5 and pVL1393-FLAG-PP5) - The 5' end of our original rat PP5 cDNA clone(Chinkers, 1994) was extended by the polymerase chain reaction (PCR) using a 5' primer that encoded an EcoRI site, an initiation codon, the FLAG epitope (DYKDDDDK), and the first five amino acids of PP5, and a 3' primer complementary to nucleotides 815-832 of this cDNA clone. The DNA fragments amplified by PCR were subcloned and sequenced from their 5' end to the NheI site at nucleotide 392 to ensure the absence of unwanted mutations. This modified DNA fragment encoding the FLAG epitope was used to replace the 5' end of the original cDNA clone up to the NheI site. The resulting construct was cloned into the EcoRI site of the pCMV6 vector (Andersson et al., 1989) for mammalian cell expression, or the pVL1393 vector for baculovirus construction.

The PP5 cDNA sequence published by Becker et al. (Becker et al., 1994), the PP5 genomic DNA sequence published by Xu *et al.* (Xu et al., 1996), and the sequence of a partial-length mouse PP5 cDNA (Chinkers, 1994) suggested that the 5' end of our rat PP5 cDNA clone was incomplete. The correct 5' sequence encodes AEGERTECAEPPRDEP PAEGTLK after the initiation codon, whereas ours encoded QGYAEGTLK . In order to correct the 5' sequence of our cDNA clone, we used oligonucleotide-directed mutagenesis

(Kunkel et al., 1987). Plasmid pBS-KS(+)-FLAG-PP5 was transformed into E.coli CJ236 (dut^- ung^- F'). Transformants were then infected with M13 helper phage to make uracil-containing single-stranded DNA. The phosphorylated oligonucleotide 5'-AAGGACGACG ATGACAAGGCCGAAGGCGAACGTACTGAATGTGCTGAACCTCCTCGAGACGAA CCTCCTGCGAAGGCACTCTGAAGC-3' was used to change the amino-terminal sequence of the FLAG-tagged construct from MDYKDDDDKQGYAEGTLK, to MDYKDDDDKAEGERTECAEPPRDEPPAEGTLK (underlined residues constitute the FLAG epitope). The phosphorylated oligonucleotide was incubated with the uracil-containing DNA template, and a buffer system containing T4 DNA polymerase, T4 DNA ligase, and nucleoside triphosphates to create heteroduplex DNA molecules. The resulting double-stranded DNA was transformed into E.coli TG-1 (dut^+ ung^+) competent cells. Uracils in the template were removed by the action of uracil N-glycosylase to produce apyrimidinic sites which are lethal lesions, so the desired mutant DNA was efficiently recovered. Sequencing of plasmids from resulting colonies was performed to confirm the mutagenesis and the absence of unwanted mutations. This corrected FLAG-tagged PP5 cDNA was subcloned into the EcoRI site of pCMV6 for the mammalian cell expression studies described below.

Plasmid expressing the FLAG-tagged TPR domain of PP5 (pCMV6-FLAG-TPR/PP5) - The corrected FLAG-tagged PP5 construct in pBS-SK(+) as described above was truncated at a unique HindIII site to remove sequences encoding the carboxyl portion of PP5. Blunt-ending with Klenow fragment and insertion of a linker encoding an NheI site and a stop codon resulted in a cDNA encoding the FLAG-tagged TPR domain of PP5, terminating at Leu^{181}. Few non-TPR residues were present in the FLAG-TPR/PP5 construct; the TPR domain begins at Ala^{28} and ends at Leu^{165} (numbering as in Becker *et al.* (Becker et al., 1994)). The cDNA fragment encoding the FLAG-tagged TPR domain of PP5 was excised as an EcoRI/SalI fragment and cloned into the corresponding sites of pCMV6 for mammalian cell expression.

Plasmid expressing the FLAG-tagged TPR domain of FKBP52 (pCMV6-FLAG-TPR/FKBP52) - The DNA fragment encoding FLAG-tagged residues 232-389 from a rabbit FKBP52 cDNA clone (Lebeau et al., 1992) was amplified by PCR using the vector pGEM 7Zf(+) containing a rabbit FKBP52 cDNA as a template, a 5' primer encoding an EcoRI site, an initiation codon, the FLAG epitope, and the residues 232-237 of FKBP52, and a 3' primer complementary to nucleotides 1153-1170. The PCR product was cloned into pBS-KS(+), and sequenced to confirm the absence of unwanted mutations. The StuI/KpnI DNA fragment from pGEM 7Zf(+)-FKBP52 was subcloned into the pBS-KS(+) containing FLAG-tagged residues from 232 to 389 in FKBP52. This resulting clone was designated pBS-KS(+)-FLAG-TPR/FKBP52, and encoded FLAG-tagged residues from 232 to the carboxyl-terminus of FKBP52. The cDNA encoding the FLAG-tagged TPR domain of FKBP52 was then excised as an EcoRI fragment and cloned into the EcoRI site of pCMV6.

Plasmid expressing the FLAG-tagged TPR domain of Hop (pCMV6-FLAG-TPR/Hop) - The DNA fragment encoding FLAG-tagged residues from 225 to 332 in a mouse Hop cDNA clone in pGEX-3X (Lassle et al., 1997) was amplified by PCR using a 5' primer encoding an EcoRI site, an initiation codon, the FLAG epitope, and residues 225 - 230 of Hop, and a 3' primer complementary to nucleotides encoding Hop residues 326-332. The PCR product was cloned into the EcoRI and HindIII sites of pBS-KS(+). The BamHI/HindIII fragment from this construct was used to replace the wild type BamHI/HindIII fragment from pGEX-3X containing the full-length Hop cDNA. This resulting construct was designated pGEX-3X-FLAG-TPR/Hop, and encoded the FLAG-tagged residues from 225 to the carboxyl-terminus of Hop. The EcoRI fragment of pGEX-3X-FLAG-TPR/Hop was subcloned into pCMV6.

Preparation of a recombinant baculovirus expressing FLAG-tagged PP5

A baculovirus for expression of FLAG-tagged PP5 was prepared by homologous recombination of pVL1393-FLAG-PP5 with BaculoGold linearized baculovirus DNA (Pharmingen) as described (Wilson and Chinkers, 1995). Plaque-purified second passage virus stocks were used for infections.

Purification of FLAG-tagged PP5 from Sf9 cells

A 100-ml suspension culture of Sf9 cells was infected with a recombinant baculovirus expressing FLAG-tagged PP5 at a multiplicity of infection of 3, then incubated for 3 days at 27°C. Cells were collected by centrifugation, washed once with 20 mM Hepes, pH 7.4, 150 mM NaCl, and sonicated in 5 ml of 20 mM Hepes, pH 7.4, 50 mM NaCl, 1 mM dithiothreitol, 20 mM benzamidine, 10 µg/ml each aprotinin, leupeptin, and pepstatin. The homogenate was centrifuged at 12,000 x g for 30 min at 4°C, and the supernatant was then centrifuged at 125,000 x g for 30 min at 4°C. The supernatant from the second centrifugation was applied to a 1-ml MonoQ column at 4°C at a flow rate of 1 ml/min. Proteins were fractionated by elution with a 20-ml gradient of 50-500 mM NaCl in 20 mM Hepes, pH 7.4, 1 mM dithiothreitol. Peak fractions containing FLAG-tagged PP5, as determined by immunoblotting with the M2 monoclonal antibody against the FLAG epitope (IBI), were pooled and further purified by immunoaffinity chromatography. The fractions were incubated batchwise with 1 ml of M2-agarose beads (IBI) for 1 h at 4°C, then transferred to a column and washed three times with 5 ml of Hepes-buffered saline containing 1 mM dithiothreitol. FLAG-tagged PP5 was then eluted by five repeated applications of 1 ml of Hepes-buffered saline, 1 mM dithiothreitol containing 200 µg/ml FLAG peptide (DYKDDDDK) for 15 min each. Fractions were analyzed by immunoblotting using the M2 antibody (IBI), and peak fractions were pooled. The FLAG peptide was then removed by ultrafiltration. Approximately 200 µg of purified protein were typically recovered from a 100-ml culture.

Preparation of rabbit antisera against PP5

After collection of preimmune serum, female New Zealand White rabbits were injected subcutaneously with 25-50 μg of purified FLAG-tagged PP5 mixed with complete Freund's adjuvant. The antigen was mixed with incomplete Freund's adjuvant for two booster injections given at 30-day intervals. Blood was collected and serum was prepared 10 days postinjection.

Transfections

DEAE-dextran-mediated transfections - COS-7 cells in 10-cm plates were transfected with 10 μg of pCMV6-FLAG-PP5, pCMV6-FLAG-TPR/PP5, or pCMV6 vector by a DEAE-dextran method (Cullen, 1987). Plasmid DNA was mixed with 1.9 ml PBS, followed by adding 0.1 ml of 10 mg/ml DEAE-dextran in PBS. Cells were washed with PBS, and incubated with this mixture at 37°C for 30 min. Ten ml of DMEM/10% FCS containing 80 μM chloroquine (Sigma) was then added to each plate, followed by incubation at 37°C for 2.5 h. Five ml of DMEM/10% FCS containing 10% DMSO was added to each plate after removing medium, followed by incubation at room temperature for 2.5 min. After aspirating medium, 10 ml of DMEM/10% FCS was added to each plate. Cells were used to perform experiments 2 days later.

Calcium phosphate transfections - For expression of the TPR domains of PP5, Hop, and FKBP52, COS-7 cells in 10-cm plates were transfected with 16 μg of pCMV6-FLAG-TPR/PP5, pCMV6-FLAG-TPR/Hop, pCMV6-FLAG-TPR/FKBP52, or pCMV6 vector using a calcium phosphate transfection kit (Gibco BRL), following the manufacturer's instructions. For studies of GR-mediated transcriptional activation, the calcium phosphate transfection procedure of Chen et al. (Chen and Okayama, 1987) was used. 2 x 10^5 CV-1 cells were seeded into a 6-cm plate, and, one day later, were transfected with 0.5 μg of pSVL-GR (Meisfeld et al., 1986), 2 μg of the CAT reporter plasmid PRE-PBL7 (Tsai et al., 1989), 0.5 μg of pRSV-luciferase (deWet et al., 1987) (to

normalize for variations in transfection efficiency), and 5 μg of total plasmid DNA containing the indicated amounts of pCMV6-FLAG-TPR/PP5, pCMV6-FLAG-TPR/FKBP52, pCMV6-FLAG-TPR/Hop and pCMV6.

Preparation of cell extracts for immunoprecipitation

Cell extracts from COS-7 cells or from L929 cells for immunoprecipitation of PP5 - L929 cells were treated with 1 μM geldanamycin or 0.1% DMSO vehicle at 37°C for 18 h. COS-7 cells were without any treatment. Cells were placed on ice, washed once with cold phosphate-buffered saline (PBS) and once with buffer A (10 mM Hepes, pH 7.4, 1 mM EDTA, 20 mM sodium molybdate, 10 mM $MgCl_2$). Cells were then scraped into buffer A containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml each aprotinin, leupeptin, and pepstatin, and lysed by passage 30 times through a 25-gauge needle. The lysate was then subjected to centrifugation at 18,500 x g for 5 min at 4°C. The supernatant from this centrifugation was clarified by centrifugation at 100,000 x g for 1 h at 4°C.

Rat brain extracts - For immunoprecipitation and immunoblotting, half of a rat brain was homogenized in 6-ml of ice cold buffer A containing 2 mM PMSF and 10 μg/ml each aprotinin, leupeptin, and pepstatin, using three 15-sec bursts with a Polytron homogenizer. The homogenate was centrifuged at 12,000 x g for 10 min at 4°C. The supernatant from this centrifugation was clarified by centrifugation at 100,000 x g for 1 h at 4°C.

Cell extracts from L929 cells for immunoprecipitation of glucocorticoid receptor and hsp90 - L929 cells were harvested by scraping into Earle's balanced salt solution followed by a wash in the same buffer and centrifugation at 500 x g. The washed cells were suspended in 1.5 volumes of 10 mM Hepes, 1 mM EDTA, 20 mM sodium molybdate, pH 7.4, and lysed by Dounce homogenization. Homogenates were centrifuged for 1 h at 100,000 x g, and the supernatant was used for immunoprecipitations.

Metabolic Labeling and Immunoprecipitation

COS-7 cells were incubated for 16 h in DMEM (without glutamine, methionine and cystine; ICN) containing 100 μCi/ml of [^{35}S]methionine/cysteine (EXPRE^{35}S^{35}S protein labeling mix, DuPont NEN). After cell extracts were normalized for total radioactivity, they were precleared by incubating for 1.5 h at 4°C with 20 μl of goat anti-mouse-IgG beads (Sigma) or protein A-agarose beads (Pierce). 2.25 μg of M2 antibody, or IgG from 5 μl of preimmune serum or anti-PP5 serum was prebound to 20 μl of goat anti-mouse IgG beads or protein A beads, respectively, by incubating antibody and beads in 100 μl of buffer A at room temperature for 1.5 h. The goat anti-mouse IgG beads or protein A beads bound to IgG were washed with 1 ml of buffer A three times. After preclearing, cell extracts were incubated for 1.5 h at 4°C with either 20 μl goat anti-mouse-IgG beads to which 2.25 μg of M2 antibody had been preadsorbed, or 20 μl of protein A-agarose beads to which the IgG from 5 μl of preimmune or anti-PP5 serum had been preadsorbed. Beads were then washed five times with 10 mM Hepes, pH 7.4, 1 mM EDTA, 20 mM sodium molybdate, 50 mM KCl, 10% glycerol, and immune complexes were released by heating in SDS sample buffer. Proteins were analyzed by SDS-PAGE and fluorography using sodium salicylate (Chamberlain, 1979). Molecular weight markers (Bio-Rad) were detected by staining with Coomassie Blue.

Immunoprecipitation and Immunoblotting

For analyzing the association of hsp90 with PP5 in COS-7 cells - Anti-PP5 immunoprecipitates were prepared from unlabeled cells as described above. The protein concentration of cell extracts was normalized before performing immunoprecipitation. Immune complexes were separated by SDS-PAGE on a 12% gel, and transferred to nitrocellulose membranes. Membranes were then blocked with TBST (20 mM Tris-HCl, pH 7.6, 138 mM NaCl, 0.1% (v/v) Tween 20) containing 5% non-fat dry milk. Immunoblotting to detect hsp90 was performed using a 1:1000 dilution of monoclonal

antibody AC-16 (Sigma) in TBST, followed by incubation with a 1:3000 dilution of peroxidase-conjugated secondary antibody (Amersham) in TBST and detection by chemiluminescence (NEN).

For analyzing the associations of hsp90, hsp70, Hop, and p23 with PP5 in L929 cells with or without geldanamycin treatment - Cell extracts with equal protein concentration (3.9 μg/ml; 660 μl) from cells treated with 1 μM geldanamycin or 0.1% DMSO vehicle at 37°C for 18 h were subjected to immunoprecipitation with preimmune or anti-PP5 serum as described above, except using 8 μl of preimmune or anti-PP5 serum, and 30 μl of protein A-beads. Following SDS-PAGE of the washed immune complexes and blotting to nitrocellulose membranes, immunoblottings to detect hsp90, hsp70, Hop, and p23 were performed as described above, except using a 1:2000 dilution of anti-hsp70 monoclonal antibody (Sigma), 1:1000 dilution of anti-Hop monoclonal antibody, and 1:2000 dilution of anti-p23 monoclonal antibody (anti-Hop and anti-p23 monoclonal antibodies were gifts from Dr. David Toft).

For analyzing the association of hsp90 with PP5 in rat brain - Equal aliquots (2.3 ml) of rat brain extracts were subjected to immunoprecipitation with preimmune or anti-PP5 serum as described above, except using 40 μl of protein A-agarose beads and 10 μl of serum. Following SDS-PAGE of the washed immune complexes and blotting to nitrocellulose filters, immunoblotting to detect hsp90 was performed as described above.

Immunoprecipitation of glucocorticoid receptor from L929 cells - The BuGR2 monoclonal antibody against glucocorticoid receptor (Affinity Bioreagents) or nonimmune mouse IgG was prebound to protein A-Sepharose beads by incubating 40 μl of a 20% slurry of protein A-Sepharose for 1 h at 4°C with 150 μl of TEG buffer (10 mM TES, 50 mM NaCl, 4 mM EDTA, 10% (w/v) glycerol, pH 7.6), and 6 μg of antibody, followed by centrifugation and washing with TEG. Glucocorticoid receptors were immunoprecipitated from 400 μl of L929 cell extract by rotation for 2 h at 4°C with 8 μl of protein A-Sepharose beads prebound with BuGR2 or nonimmune mouse IgG, followed by three washes with 1

ml of TEGM buffer (TEG plus 20 mM sodium molybdate for stabilizing glucocorticoid receptor heterocomplexes). Immune complexes were fractionated by SDS-PAGE on a 12% gel, transferred to Immunobilon-P membranes, and probed with 1 μg/ml BuGR2 antibody for glucocorticoid receptor, 1 μg/ml of monoclonal antibody AC88 (StressGen) for hsp90, 1000-fold dilutions of anti-PP5 serum, an antiserum to the C-terminal peptide of CyP-40 (Affinity Bioreagents), or an antiserum designated UPJ56 raised against FKBP52 (Ruff et al., 1992), provided by Karen Leach (The Upjohn Co.). The immunoreactive bands were visualized by incubation with ^{125}I-conjugated secondary antibody and autoradiography.

Immunoprecipitation of hsp90 from L929 cell extracts - L929 cell extracts were diluted 10-fold in 10 mM Hepes, pH 7.4, 1 mM EDTA, 10% glycerol, and 100-μl aliquots were incubated with 10-μl pellets of Actigel-ALD precomplexed with either nonimmune IgM or with 3G3 anti-hsp90 monoclonal antibody (Affinity Bioreagents). Immune pellets were washed three times in 1 ml of TEGM buffer, and both immune pellets and the immunoadsorbed extracts were assayed by Western blotting for hsp90 and PP5, and by autoradiography, as described above.

Gel filtration chromatography

A rat brain extract was freshly prepared essentially as described above, but in a reduced volume of the lysis buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 3 mM $MgCl_2$, 50 mM KCl), and passed through a 0.2 μm filter. 200 μl of the filtered extract, containing approximately 5.6 mg of protein, was then loaded onto a 25-ml Superose 6 column equilibrated with lysis buffer. The column was previously calibrated with the following standards, using the same buffer: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and ovalbumin (43 kDa). Samples were eluted with the same buffer at a flow rate of 0.2 ml/min, and 0.5-ml fractions were collected. 40 μl of each fraction were analyzed by SDS-PAGE in 8.5% gels, followed by immunoblotting and detection with

anti-PP5 (1:4000) or with monoclonal antibody AC-16 against hsp90 (1:1000) and chemiluminescence, as described above.

Glucocorticoid-induced transcriptional activation

CV-1 cells were transfected as described above, incubated in the absence or presence of 100 nM dexamethasone for 26 h, washed twice with PBS, harvested by scraping into PBS, and collected by centrifugation at 4°C for 3 min at 7,500 x g. Cells were then resuspended in 100 µl of 0.1 M potassium phosphate, pH 7.8, 1 mM dithiothreitol, and lysed by three cycles of freezing in an ethanol/dry ice bath and thawing in a 37°C water bath. After clarifying the lysate by centrifugation at 4°C for 5 min at 18,500 x g, 85 µl of the supernatant were saved and diluted for luciferase (deWet et al., 1987) and CAT (Seed and Sheen, 1988) assays. To inactivate endogenous acetylating enzymes, cell extracts were heated at 65°C for 15 min before performing CAT assays. To correct for variations in transfection efficiency, CAT activity was normalized to luciferase activity. Luciferase and CAT activity were linear with respect to time and protein concentration.

Cell fractionation and immunoblotting

HeLa or L929 cells in monolayer culture were harvested by washing with PBS, and incubating with trypsin-EDTA solution for 5 min at 37°C. The cell suspension was then washed with DMEM/10% FCS to inhibit trypsin, and centrifuged at 1,400 x g for 5 min. Cell pellets were washed with 3 ml of lysis buffer (10 mM Hepes, pH 7.4; 1.5 mM $MgCl_2$; 10 mM KCl; 2 mM PMSF; 10 µg/ml each aprotinin, leupeptin, and pepstatin), and resuspended in 2 ml of lysis buffer. 3.0×10^7 HeLa cells or 3.5×10^7 L929 cells in 1.5 ml of lysis buffer were homogenized with a 7-ml Dounce homogenizer with a B-pestle, using 50 strokes. Cells were stained with trypan blue and examined under a microscope to ensure that almost all of the cells were lysed. 750 µl of lysate was centrifuged at 7,500 x g for 5 min. The supernatant was collected as the cytoplasmic fraction. The nuclear pellet was

suspended in 750 µl of lysis buffer. This suspension was sonicated three times on ice, each time for 15 sec, and cooled on ice for 1 min between sonications. This sonicated suspension was designated the nuclear fraction. 28 µl of the cytoplasmic and nuclear fractions of HeLa cells, or 12 µl of the cytoplasmic and nuclear fractions of L929 cells were mixed with 7 µl or 3 µl of 5 x SDS sample buffer, respectively, and loaded onto an 8.5% gel. The separated proteins were transferred to a nitrocellulose membrane. Immunoblotting to detect PP5 was performed as described above using a 1:2000 dilution of anti-PP5 serum.

Purification of IgG

A column containing 1 ml of protein A beads (Pierce) was washed with 5 ml of binding buffer (0.1 M sodium acetate, pH 7.5; 0.5% Tween 20). One-half ml of anti-PP5 serum or preimmune serum, mixed with 0.5 ml of 0.1 M sodium acetate, pH 7.5, and 1% Tween 20 was applied to this column. The column was then washed with 7 ml of binding buffer and 3 ml of 0.1 M sodium acetate, pH 7.5. IgG was eluted with 9 ml of 0.58% (v/v) acetic acid; 150 mM NaCl, and 1-ml fractions were collected. Each 1 ml of elute was immediately neutralized with 0.5 ml of 3 M sodium acetate, pH 11.2. The fractions with high absorbance at 280 nm were collected and dialyzed overnight against 2 l of 20 mM Hepes, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$. Purified IgG was concentrated using a Centricon 30 filter (Amicon). The protein concentration of IgG from nonimmune serum was 0.8 mg/ml, and from immune serum was 1 mg/ml. The aliquots of purified IgG were stored at -70°C.

Indirect immunofluorescence

Cells fixed with acetone (-20°C) - 4 x 10^4 cells per well were seeded in a 24-well-plate containing glass cover slips on the day before experiments. Cells in each well were washed twice with PBS, and then fixed with 0.5 ml of acetone (-20°C) at -20°C for 10 min. The fixed cells in each well were washed three times with 1 ml of PBS, and

permeabilized with 200 µl of permeabilization solution (0.1 M Tris-HCl, pH 7.5; 1M NaCl; 0.5 % Triton X-100; 3% BSA; 1% normal goat serum (Sigma)) at room temperature for 10 min. 200 µl of permeabilization solution containing 2 µg/ml of purified IgG (for COS-7 cells), 1.5 µg/ml (for L929 cells), or 1 µg/ml (for HeLa cells) was added into each well, and incubated at 37°C with a humidifying system for 1 h. Cells were washed with 1 ml of washing solution (0.1 M Tris-HCl, pH 7.5; 1 M NaCl; 0.5% Triton X-100) at room temperature three times for 15 min. Cells were then incubated for 1 h with 200 µl of a 1:6000 dilution of goat anti-rabbit-IgG conjugated with rhodamine (Pierce, No. 31672) in permeabilization solution at 37°C, with a humidifying system. Cells in each well were washed with 1 ml of washing solution three times as above. The cover slips were mounted with mounting solution (as described below), and the cells were examined under a fluorescence microscope (Leica DMRB).

Mounting solution: 2.4 g of MOWIOL 04-88 (Calbiochem, No. 475904) was added to 6 g of glycerol and 6 ml of distilled H_2O. This solution was stirred for 2 h at room temperature, and then 12 ml of 0.2 M Tris-HCl, pH 8.5 was added, followed by incubation at 50°C for 10 min with occasional stirring. After centrifugation at 5,000 x g for 15 min at room temperature, aliquots of the supernatant were stored at 4°C.

In order to test the specificity of anti-PP5 IgG for immunofluorescence staining, 2.4 µg of ant-PP5 IgG was preadsorbed with 4.8 µg of purified FLAG-tagged PP5 or with 4.8 µg of the purified catalytic subunit of protein phosphatase 1 (a gift from Dr. Thomas Soderling) in 16 µl of PBS at 4°C overnight. This solution was then diluted with permeabilization solution to contain the amounts of IgG indicated above for the various cell types.

Cells fixed with 2% paraformaldehyde - After cells were washed with PBS, they were fixed with 2% paraformaldehyde in PBS (the pH was adjusted to about 8 with 1 N NaOH) at room temperature for 30 min. Subsequent procedures were as described above, except that a 1:9000 dilution of goat anti-rabbit IgG conjugated with rhodamine was used as the secondary antibody.

Results

Hsp90 co-immunoprecipitates with the overexpressed TPR domain of PP5

In an attempt to identify proteins interacting with the TPR domain of PP5, the FLAG-tagged TPR domain of PP5 was overexpressed in COS-7 cells and co-immunoprecipitation experiments were performed. A 90-kDa protein co-immunoprecipitated with the FLAG-tagged TPR domain of PP5 from [^{35}S]methionine/cysteine-labeled cells, using a monoclonal antibody to the FLAG epitope (Fig. 3A, lane 2). The 90-kDa protein was abundant enough to be stainable by Coomassie Blue (data not shown). The anti-FLAG antibody did not immunoprecipitate the 90-kDa protein in the absence of the FLAG-tagged TPR domain of PP5 (Fig. 3A, lane 1). Thus, the 90-kDa protein was a major cellular protein that was immunoprecipitated by virtue of its binding to the TPR domain of PP5.

The TPR domain of PP5 is most closely related to TPR domains found in Hop and several large immunophilins known to associate with hsp90 (Chinkers, 1994). Hop and the large immunophilins have been shown to bind to hsp90 through their TPR domains (Lassle et al., 1997; Owens-Grillo et al., 1996; Radanyi et al., 1994; Ratajczak and Carrello, 1996). Based on these observations and on the fact that hsp90 is an abundant protein, I hypothesized that the 90-kDa protein co-immunoprecipitated with the TPR domain of PP5 might be hsp90. In order to test this hypothesis, a monoclonal antibody to hsp90 was used to perform immunoblotting after immunoprecipitations using the anti-FLAG antibody. This experiment confirmed that hsp90 was co-immunoprecipitated with the FLAG-tagged TPR domain of PP5 from extracts of unlabeled COS-7 cells overexpressing this domain, but not from extracts of cells transfected with control plasmid (Fig. 3B, lane 1 and 2).

Hsp90 co-immunoprecipitates with overexpressed full-length PP5

In order to test if the interaction between the TPR domain of PP5 and hsp90 was an artifact of truncating PP5, FLAG-tagged full-length PP5 overexpressed in COS-7 cells was

immunoprecipitated with the M2 anti-FLAG antibody either from ^{35}S-labeled (Fig. 3A, lane 3) or unlabeled cells (Fig. 3B, lane 3). A 90-kDa protein from the ^{35}S-labeled cell extract was co-immunoprecipitated with FLAG-tagged full-length PP5 (Fig. 3A, lane 3), and I confirmed that this protein was hsp90 by immunoblotting using a monoclonal antibody to hsp90 (Fig. 3B, lane 3). Thus, hsp90 associates with full-length overexpressed PP5 as well as with the recombinant TPR domain.

Hsp90 co-immunoprecipitates with endogenous PP5

It was possible that the interactions with hsp90 observed above were artifacts of PP5 overexpression. In order to test this possibility, I examined whether hsp90 co-immunoprecipitated with endogenous PP5. The recombinant FLAG-tagged PP5 was purified to homogeneity by Mono Q chromatography followed by immunoaffinity chromatography on a matrix containing the M2 antibody to the FLAG epitope (Fig. 4). A specific, high titer anti-PP5 serum was made using the purified recombinant FLAG-tagged rat PP5 to immunize rabbits. Endogenous PP5, from untransfected COS-7 cells or rat brain, was then immunoprecipitated. A 90-kDa protein from ^{35}S-labeled untransfected COS-7 cells co-immunoprecipitated with endogenous PP5 (Fig. 5A), and was confirmed to be hsp90 by immunoblotting (Fig. 5B). In the rat brain extract, hsp90 also co-immunoprecipitated with endogenous PP5 (Fig. 5B). Neither PP5 nor hsp90 was immunoprecipitated by preimmune serum (Fig. 5A, B). Thus, endogenous PP5 forms a complex with hsp90 in both cultured cells and in a normal tissue.

The reciprocal co-immunoprecipitation experiment using an antibody to hsp90 was performed to confirm the association between PP5 and hsp90. PP5 from an extract of mouse L929 cells co-immunoprecipitated with hsp90 using a monoclonal IgM to hsp90 (Fig. 6, lane 4), but not using nonimmune IgM (Fig. 6, lane 3). Thus, just as hsp90 co-immunoprecipitates with PP5, PP5 also co-immunoprecipitates with hsp90.

The majority of PP5 is in the cytoplasm

It was initially reported that PP5 was primarily a nuclear protein in normal human fibroblasts, HeLa cells, and A431 cells, although some PP5 was detected in the cytoplasm (Chen et al., 1994). If true, the results shown above would be somewhat surprising, since hsp90 is primarily a cytoplasmic protein (Gasc et al., 1990; Lai et al., 1984). I therefore re-examined the subcellular localization of PP5 in HeLa and L929 cells using the methods of Chen *et al.* (Chen et al., 1994). Cytoplasmic and nuclear fractions were prepared as described in Materials and Methods, and examined by immunoblotting using an anti-PP5 serum. Although nuclear PP5 was detectable, the majority of PP5 in both HeLa and L929 cells was in the cytoplasmic fraction (Fig. 7). This suggested that PP5 and hsp90 were co-localized in the cytoplasm, consistent with the possibility that they interact each other *in vivo*.

It was possible that PP5 leaked out of the nucleus during subcellular fractionation. Therefore, the subcellular localization of PP5 was also examined by indirect immunofluorescence using purified IgG from preimmune serum or anti-PP5 serum. The subcellular localization of proteins examined by indirect immunofluorescence may vary depending on the fixation method, so both 2% paraformaldehyde (Fig. 8) and acetone (-20°C) (Fig. 9) were used for cell fixation. The first method was used by Chen *et al.* (Chen et al., 1994), when they reported nuclear staining of PP5. The immunofluorescence patterns of PP5 in HeLa, L929, and COS-7 cells were similar, whether cells were fixed with 2% paraformaldehyde or acetone (-20°) (Figs. 8 and 9). Most PP5 was in the cytoplasm (Figs. 8 and 9, I). This was not due to non-specific staining, because PP5 was not stained by preimmune IgG (Figs. 8 and 9, PI). Moreover, preadsorption of anti-PP5 IgG with purified PP5 largely blocked staining (Figs. 8 and 9, I+PP5). The PP5 immunofluorescence pattern was unaltered by preadsorption of anti-PP5 IgG with the catalytic subunit of protein phosphatase 1 (PP1C) (Figs. 8 and 9, I+PP1C). Thus, cytoplasmic staining was apparently not due to cross-reaction of our antibody with other

protein phosphatases. These results suggest that most PP5, like most hsp90, is in the cytoplasm. This is consistent with the two proteins interacting *in vivo*.

PP5 and hsp90 are present in a high molecular weight complex

Gel filtration was used as an alternative approach to confirm the association between PP5 and hsp90. Rat brain extract was fractionated on a Superose 6 column calibrated with various molecular weight markers. Fractions were then analyzed by SDS-PAGE followed by immunoblotting with an anti-PP5 serum or with a monoclonal antibody to hsp90 (Fig. 10). In addition to a monomeric peak of PP5, much of the PP5 in rat brain extract co-migrated with hsp90 in a peak of approximately 600 kDa. This result was consistent with the association between PP5 and hsp90 demonstrated in co-immunoprecipitation experiments, and suggested that both molecules were present in a large complex.

Since steroid receptors exist as large heterocomplexes containing hsp90 (Pratt, 1993; Smith and Toft, 1993), and TPR proteins bound to hsp90 (Pratt and Toft, 1997), I hypothesized that PP5 might be a component of steroid receptor complexes. I tested this hypothesis using glucocorticoid receptor (GR) as a model system.

PP5 co-immunoprecipitates with the glucocorticoid receptor

It was tested whether PP5 was associated with GR heterocomplexes using a co-immunoprecipitaion approach. L929 cell extracts were subjected to immunoprecipitation using a monoclonal antibody to GR (Fig. 11, lane 3) or a nonimmune control antibody (Fig. 11, lane 2). Immunoblotting was then performed using antibodies to GR, hsp90, PP5, FKBP52 or CyP-40, followed by ^{125}I-conjugated secondary antibody. The sample in lane 1 (Fig. 11) was the original L929 cell extract before immunoprecipitation. As shown previously (Owens-Grillo et al., 1996; Renoir et al., 1990; Rexin et al., 1991), hsp90, FKBP52, and CyP-40 co-immunoprecipitated with GR (lane 3). In addition, PP5

co-immunoprecipitated with GR (lane 3). Thus, PP5 is a component of GR heterocomplexes.

The TPR domain of PP5 acts as a dominant negative mutant to block GR-mediated transactivation

The above experiments demonstrated that PP5 was a component of GR heterocomplexes with hsp90. I next asked whether PP5 regulated stimulation of gene expression by glucocorticoids. To test this hypothesis, a deletion mutant of PP5 containing the TPR domain, but lacking the phosphatase catalytic domain, was used as a dominant negative mutant to displace endogenous PP5 from hsp90 in GR heterocomplexes.

The TPR domain of PP5 was co-expressed in CV-1 cells with GR, a CAT reporter gene under the control of glucocorticoid response elements, and luciferase. Dexamethasone treatment induces CAT expression through dexamethasone-GR complexes binding to the hormone response elements in the reporter plasmid. CAT activity in cells with or without dexamethasone treatment was normalized to luciferase activity to control for variation in transfection efficiency. Dexamethasone-induced CAT activity in the absence of the TPR domain of PP5 was considered 100%. As shown in Fig. 12, the TPR domain of PP5 inhibited GR-mediated transactivation in a concentration-dependent manner, and dexamethasone-induced gene expression was inhibited by approximately 65% at the highest concentration of pCMV6-FLAG-TPR/PP5 tested. This result suggested that PP5 was required for optimal GR-mediated transactivation.

In GR heterocomplexes, Hop, and various large immunophilins (FKBP51, FKBP52, and CyP-40) also contain TPR domains, and all of these proteins appear to compete for the same TPR acceptor site on hsp90 *in vitro* (Silverstein et al., 1997). It was possible that the effect of the PP5 TPR domain in Fig. 12 was due to the displacement of these TPR-proteins from GR heterocomplexes, rather than to specific displacement of PP5. To test

this possibility, the effects of the TPR domains of Hop and FKBP52 on GR-mediated transactivation were examined.

As shown in Fig. 13A, the FLAG-tagged TPR domains of PP5, Hop, and FKBP52 were expressed with correct molecular weights in COS-7 cells. The effects of these TPR domains on GR-mediated transcriptional activation were then examined. The FLAG-tagged TPR domains of PP5, Hop, or FKBP52 were co-expressed with plasmids encoding a CAT reporter gene, GR, and luciferase in CV-1 cells. Dexamethasone-induced CAT activity in the absence of plasmids expressing TPR domains was considered 100%. The TPR domain of PP5 inhibited GR-mediated transactivation by approximately 85% (Fig. 13B). In contrast, the TPR domains of Hop and FKBP52 did not significantly inhibit GR-induced transactivation. This suggested that the results in Fig. 12 were due to specific displacement of PP5 from GR heterocomplexes. Thus, PP5 appears to be important for signaling by the GR. The mechanisms by which PP5 modulates GR signaling remain to be elucidated.

PP5 is in the heterocomplex containing Hop and the heterocomplex containing p23

Although it was shown that PP5 co-immunoprecipitated with GR (Fig. 11), it is not clear at which stage PP5 is involved in GR heterocomplex formation. The model for the steroid receptor heterocomplex assembly pathway is (Fig. 1): the receptor-hsp70-Hip complex (an early heterocomplex) --> the receptor-hsp70-Hip-Hop-hsp90 complex (an intermediate heterocomplex) --> the receptor-hsp90-p23-large immunophilin complex (a mature heterocomplex). In order to address this question, co-immunoprecipitation experiments were performed using preimmune (Fig. 14, PI) or anti-PP5 serum (Fig. 14, I), followed by immunoblotting using an antibody to hsp90, hsp70, Hop, or p23. Besides hsp90, hsp70, Hop, and p23 specifically co-immunoprecipitated with PP5 using an anti-PP5 serum. The preimmune serum did not immunoprecipitate hsp90, hsp70, Hop, or p23. This suggested that PP5 was present in both intermediate and mature complexes.

Geldanamycin affects the formation of receptor heterocomplexes by increasing recovery of the intermediate heterocomplex and eliminating the mature heterocomplex (Smith et al., 1995), so it was used as a tool to further test for the presence of PP5 in intermediate complexes. The amount of hsp90 co-immunoprecipitated with PP5 was the same in the cells with or without geldanamycin treatment. P23 co-immunoprecipitated with PP5 in the cells without geldanamycin treatment, but not in the cells treated with geldanamycin. The amounts of hsp70 and Hop co-immunoprecipitated with PP5 in the cells treated with geldanamycin were about 4-fold higher than that in the cells without geldanamycin treatment. These results suggested that PP5 was in both the intermediate heterocomplex and the mature heterocomplex. It has been reported that PP5 and large immunophilins exist in different steroid receptor heterocomplexes (Silverstein et al., 1997). Based on this information and my results, there are two possibilities for the steroid receptor heterocomplex assembly pathway including PP5. One possibility is that PP5 and large immunophilins are in the same pathway: a receptor-hsp70-Hip-Hop-hsp90-PP5 complex --› a receptor-hsp90-PP5-p23 complex --› a receptor-hsp90-p23-large immunophilin complex. The other possibility is that PP5 and large immunophilins are in different pathways: a receptor-hsp70-Hip-Hop-hsp90-PP5 complex --> a receptor-hsp90-PP5-p23 complex; a receptor-hsp70-Hip-Hop-hsp90 complex --> a receptor-hsp90-p23-large immunophilin complex. Thus, the role of PP5 in the steroid receptor heterocomplex assembly pathway needs to be further addressed.

Discussion

Mounting evidences suggest that TPR domains mediate protein-protein interactions. The TPR domain of PP5 has been shown to bind to the protein kinase-like domain of the ANP receptor in the yeast two-hybrid system and *in vitro*, suggesting that PP5 may be the protein phosphatase that dephosphorylates and desensitizes the ANP receptor (Chinkers, 1994). In general, the protein serine/threonine phosphatases in the PPP family have multiple functions (Barford, 1996; Faux and Scott, 1996; Hubbard and Cohen, 1996). Thus, it seemed likely that the TPR domain of PP5 interacted with proteins other than the ANP receptor.

Co-immunoprecipitation experiments showed that hsp90 was bound to the overexpressed TPR domain of PP5. Moreover, hsp90 was shown to bind to endogenous PP5. Thus, the interaction between the TPR domain of PP5 and hsp90 is not an artifact due to truncation of PP5 or overexpression. It is not clear if all four TPRs in the TPR domain of PP5 are required for this interaction. The TPR domain of PP5 is most closely related to the TPR domains of the large immunophilins (FKBP52 and CyP-40) and Hop (Chinkers, 1994). It has been shown that the TPR domains (containing 3 TPRs) of FKBP52 and CyP-40 were necessary but not sufficient for association with hsp90. Flanking acidic and basic residues clustered at the amino- and carboxyl-terminal ends of the TPR domain were required for binding to hsp90 (Radanyi et al., 1994; Ratajczak and Carrello, 1996).

Indirect immunofluorescence and cellular fractionation experiments showed that the majority of PP5 is in the cytoplasm, although there is some PP5 in the nucleus. The subcellular localization of hsp90 is primarily in the cytoplasm (Gasc et al., 1990; Lai et al., 1984). Like PP5, some hsp90 is also present in the nucleus. The similarity between the subcellular localization of PP5 and hsp90 supports that hsp90 interacts with PP5 *in vivo*. Cohen and her colleagues reported that PP5 was primarily a nuclear protein (Chen et al., 1994). These apparently contradictory results may be due to the different antibodies used

for these experiments, because I used the same fixation method (2% paraformaldehyde), washing conditions, and cell line (HeLa cells) that they used for indirect immunofluorescence. In order to confirm the anti-PP5 staining was specific, I preincubated the anti-PP5 IgG with purified PP5 to block specific staining or with the purified catalytic subunit of PP1 to block non-specific staining. All of these results indicated that the staining from my purified anti-PP5 IgG was specific. The acetone (-20°C) fixation method for indirect immunofluorescence also showed that the majority of PP5 was in the cytoplasm. According to immunoblotting, the anti-PP5 serum I used was specifically against PP5. Thus, my results suggest that PP5 is primarily located in the cytoplasm, not in the nucleus. I speculate that the structure of PP5 and/or the proteins associated with PP5 in the nucleus is different from that in the cytoplasm. Thus, Cohen's antibodies could be more efficiently directed against the PP5 in the nucleus than in the cytoplasm. According to Patricia Cohen (personal communication), subsequent experiments using other antibodies have shown that significant amounts of PP5 are present in the cytoplasm, unlike their published experiments (Chen et al., 1994), which can no longer be reproduced. The explanation for the difference in the cellular fractionation results between our two laboratories is that cytoplasmic and nuclear fractions were normalized differently. Chen *et al.* (Chen et al., 1994) loaded 2 µl of the cytoplasmic and 10 µl of the nuclear fraction onto the SDS-PAGE for immunoblottings, with the nuclear pellet being resuspended in the same volume as the cytoplasmic fraction. I also prepared cytoplasmic and nuclear fractions in the same volume, but loaded equal volumes of both fractions to the SDS-PAGE for immunoblottings.

There is a result that indirectly supports my indirect immunofluorescence and cellular fractionation results. Skinner *et al.* purified PP5 as a protein phosphatase which is activated by arachidonic acid and is thought to regulate ion channels (Skinner et al., 1997). Because a soluble protein phosphatase modulating ion channels would be expected to be cytoplasmic, PP5 would be expected to be a cytoplasmic protein.

Gel filtration was used as an alternative approach to confirm the association between PP5 and hsp90. In addition to a peak representing free PP5, much of the PP5 co-migrated with hsp90 in a peak of approximately 600 kDa, consistent with the association between PP5 and hsp90 demonstrated in co-immunoprecipitation experiments. It is not clear if all of PP5 interacts with hsp90 *in vivo*. This free PP5 population may be created during experimental manipulation, or may represent a free PP5 population occurring *in vivo*. If there is some PP5 which does not interact with hsp90 *in vivo*, then some PP5 functions would not involve hsp90. Most hsp90 has been reported to be dimeric *in vivo* (Perdew et al., 1993). I did not detect dimeric hsp90, but monomeric hsp90 was seen in one of three experiments. Hsp90 is a major protein in the cell, but PP5 is not. Thus, not all hsp90 can be bound to PP5 *in vivo*.

The gel filtration results suggest that PP5 and hsp90 co-exist in a 600-kDa complex. The size of the [^{3}H]steroid-labeled cross-linked molybdate-stabilized steroid receptor heterocomplex, determined by gel filtration under different experimental conditions was 300-350 kDa, and this heterocomplex contained 1 molecule of receptor, 2 molecules of hsp90, and 1 molecule of the large immunophilins (Rehberger et al., 1992; Rexin et al., 1991; Segnitz and Gehring, 1995). The difference between my gel filtration result and these reports may be due to the difference in the experimental method and condition. Alternatively, my gel filtration results raise several possibilities: 1. There is a large steroid receptor heterocomplex containing hsp90 and PP5 which has not previously been described, 2. my gel filtration conditions make steroid receptor heterocomplexes aggregate, or 3. PP5 and hsp90 associate with proteins other than those found in steroid receptor heterocomplexes. It has been reported that the 8S GR heterocomplex binds to actin filaments through hsp90 (Miyata and Yahara, 1991), so additional proteins can indeed associate with these heterocomplexes.

PP5 is associated with GR, implying that PP5 plays a role in the GR signaling pathway. The TPR domain of PP5 is able to act as a dominant negative mutant to inhibit

GR-mediated gene expression *in vivo*, further suggesting that PP5 may be important in the GR signaling pathway. However, besides PP5, Hop and the large immunophilins (FKBP51, FKBP52, and CyP-40) in the steroid receptor heterocomplex contain TPR domains and they interact with hsp90 through their TPR domains. This raised the possibility that the effect of the TPR domain of PP5 on the GR-mediated gene expression could be due to the displacement of these other TPR-proteins, in addition to PP5, from the GR heterocomplex. Arguing against this, the Hop and FKBP52 TPR domains, unlike the TPR domain of PP5, were not efficient in inhibiting GR-mediated gene expression in CV-1 cells (Fig 13B). This result is somewhat confusing, since it has been reported that Hop is required for steroid receptor heterocomplex formation *in vitro* (Dittmar et al., 1996), and deletion of the gene of Sti1, the yeast Hop homologue, reduces GR-mediated transactivation activity in yeast cells (Chang et al., 1997). It is possible that the expression of the Hop TPR domain was too low to displace endogenous Hop. The expression of this TPR domain was much lower than that of the TPR domain of PP5 in COS-7 cells, as was the expression of the FKBP52 TPR domain, which also did not inhibit GR-mediated gene expression. However, the levels of all 3 TPR domains are too low to measure in transfected CV-1 cells, so that I can not say whether some expressed better than others in the transactivation experiments.

The role of large immunophilins in the steroid receptor signaling pathway is not yet defined. The immunosuppressant rapamycin inhibits the PPIase activity of FKBP52, but does not affect the chaperone activity of FKBP52 (Bose et al., 1996), suggesting that the chaperone activity of FKBP52 is independent of its PPIase activity and is contributed by the other part of FKBP52. The FKBP52 TPR domain construct contained the TPR domain (residues 273-389) and the whole carboxyl-terminus beside the TPR domain. I speculate that this construct may still be functional, because the FKBP52 TPR domain did not inhibit GR-mediated transcriptional activation. The other possibilities are that the expression of the FKBP52 TPR domain is too low to displace endogenous FKBP52, or FKBP52 is not

required for the GR signaling pathway in mammalian cells, like Cpr6 (a yeast CyP-40 homologue) in the yeast cells. Although the inhibition of GR-mediated gene expression by the TPR domain of PP5 does not definitively prove that PP5 is required for GR-mediated gene expression, it suggests that PP5 plays a role in the GR signaling pathway.

PP5 is a component of a GR heterocomplex. Besides hsp90, hsp70, Hop, and p23 co-immunoprecipitated with PP5. The associations between these proteins and PP5 are specific, according to the effect of geldanamycin on the steroid receptor heterocomplex formation. Geldanamycin treatment increases the recovery of the steroid receptor heterocomplex containing hsp70 and Hop, but eliminates the steroid receptor heterocomplex containing p23. Consistent with this, more hsp70 and Hop co-immunoprecipitated with PP5 from cells treated with geldanamycin than from cells without geldanamycin treatment, and p23 did not co-immunoprecipitate with PP5 in the treated cells. The model hypothesized for the steroid receptor heterocomplex formation pathway is (Fig. 1): the receptor-hsp70-Hip complex (an early heterocomplex) --> the receptor-hsp70-Hip-Hop-hsp90 complex (an intermediate heterocomplex) --> the receptor-hsp90-p23-large immunophilin complex (a mature heterocomplex). Hop and p23 exist in the intermediate and mature heterocomplex, respectively. Based on this information and my result, PP5 exists in the intermediate heterocomplex containing Hop, and the mature heterocomplex containing p23. The mature heterocomplex, besides containing p23, also contains one of the large immunophilins (FKBP51, FKBP52, and CyP-40). It has been shown that PP5 does not co-immunoprecipitate with FKBP52 or CyP-40 in the reticulocyte lysate and the TPR domain of PP5 competes with PP5, FKBP52, and CyP-40 for hsp90-binding *in vitro*, suggesting that PP5, FKBP52, and CyP-40 exist in different heterocomplexes with hsp90 (Silverstein et al., 1997). Taking these results together, there are two possibilities for the steroid receptor heterocomplex formation pathway including PP5. One possibility is that PP5 associates with steroid receptor heterocomplexes before large immunophilins. The other possibility is that there exist different mature heterocomplexes which contain either a

large immunophilin or PP5. It has been reported that a low level of Hop co-immunoprecipitates with p23 in reticulocyte lysate, and in the presence of molybdate, more Hop co-immunoprecipitates with p23 (Johnson and Toft, 1995). Based on this information and my result, I speculate that there exists a new pathway in the heterocomplex assembly pathway excluding large immunophilins: a hsp70-Hip-Hop-hsp90-PP5 complex --> a hsp70-Hip-Hop-hsp90-PP5-p23 complex --> a hsp90-PP5-p23 complex.

Although the regulation of PP5 activity is not fully understood, PP5 was activated by proteolytic cleavage of the TPR domain (Chen and Cohen, 1997), suggesting that the TPR domain suppresses PP5 activity. Moreover, the activity of PP5 was stimulated by pharmacological concentrations of arachidonic acid and other unsaturated fatty acids (Skinner et al., 1997). This requirement for very high, nonphysiological concentrations of unsaturated fatty acids suggested the existence of a more potent lipid or other endogenous activator. I have shown that PP5 binds to hsp90 through its TPR domain *in vivo*. This raises the possibility that hsp90 may be an endogenous activator of PP5. Alternatively, it is possible that the chaperone and ATPase activities of hsp90 are modulated by PP5, since hsp90 is a phosphoprotein. PP5 contains three multifunctional calmodulin-dependent protein kinase II/S6 kinase II consensus motifs (Kemp and Pearson, 1990) (one in the TPR domain; two in the catalytic domain), suggesting that PP5 activity could be regulated by these kinases. It has been reported that hsp90 contains a calmodulin-binding domain (Minami et al., 1993), raising the possibility that a multifunctional calmodulin-dependent protein kinase II binds to hsp90 and phosphorylates and activates PP5. While the FLAG-tagged PP5 overexpressed in COS-7 cells labeled with ^{32}P was not phosphorylated (data not shown), the multifunctional calmodulin-dependent protein kinase II and S6 kinase II are activated by extracellular signals. It remains possible that PP5 is phosphorylated and activated by these protein kinases after their activation by extracellular signals.

The classic steroid receptor family includes the GR, PR, ER, AR, and MR. The formation of the GR and PR heterocomplexes has been most extensively studied. The ER,

AR, and MR, like GR and PR, also form heterocomplexes by association with the proteins found in the GR and PR heterocomplexes. I have shown here that PP5 is a component of the GR heterocomplex. I have also tested if PP5 is a component of the PR heterocomplex by co-immunoprecipitation. In these experiments PP5 co-immunoprecipitated with the PR (data not shown), suggesting that PP5 is a component of the PR heterocomplex. Moreover, another laboratory used our anti-PP5 serum for immunoblotting to show that PP5 co-immunoprecipitated with the AR. Taking these results together, PP5 is likely to be a component of steroid receptor heterocomplexes in general.

In the steroid receptor heterocomplex, receptor, hsp90, and p23 are phosphoproteins. It has been proposed that the activation of steroid receptor requires dephosphorylation of protein(s) in the heterocomplex (Gruol and Wolf, 1989). This thesis shows that PP5 is a component of glucocorticoid receptor heterocomplexes, supporting this hypothesis. To date, the proteins associating with the steroid receptor either contain chaperone activity (e.g. hsp70, hsp90, p23, FKBP52, and CyP-40) or are co-chaperones (e.g. hsp40, Hip, and Hop which assist hsp70 chaperone activity). No evidence yet indicates whether or not PP5 has chaperone activity or functions as a co-chaperone to regulate the chaperone(s) associated with PP5. The mRNA level of hsp70, hsp90, hsp40, Hop and FKBP52 can be induced by heat shock treatment (Lassle et al., 1997; Lindquist and Craig, 1988; Ohtsuka, 1993; Sanchez, 1990). In my hands, PP5 mRNA levels are not induced by heat shock treatment, so that PP5 does not appear to be a stress-inducible protein. It has been reported that the concentration of PP5 in the cells, as judged by immunofluorescence, drops significantly when the cells are confluent, and serum deprivation also decreases the PP5 level (Chen et al., 1994) Thus, the expression of PP5 is regulated by cell growth conditions.

The findings of this thesis point out that PP5 is a hsp90-associated protein, and is a component of the steroid receptor heterocomplex. It raises the possibility that PP5 may not just be involved in the heterocomplex containing steroid receptor and hsp90, but also in the

other heterocomplexes containing hsp90. The TPR domain of PP5 acts as a dominant negative mutant to inhibit the GR-mediated gene expression *in vivo*, suggesting a role for PP5 and/or other TPR-proteins associating with the GR *in vivo*. This is the first *in vivo* evidence that these steroid receptor-associated TPR proteins are important for steroid receptor signaling and it led us to re-think the steroid receptor signaling pathway. Moreover, PP5 appears to be a component of both intermediate and mature steroid receptor heterocomplexes, which provides a more detailed understanding of the steroid receptor heterocomplex assembly pathway.

There are several questions derived from this thesis study, which will require further study. How is PP5 involved in the steroid receptor assembly pathway? What protein(s) in the steroid receptor may be the substrate(s) for PP5? How is PP5 phosphatase activity modulated? Does PP5 have chaperone activity or function as a co-chaperone? Is PP5 phosphorylated by multifunctional calmodulin-dependent protein kinase II or other protein kinases *in vivo*? How is the expression of PP5 regulated by cell growth? Does PP5 associate with other proteins, besides hsp90? As future studies answer these questions, we will better understand the physiological function of PP5, the mechanisms of the hsp90 chaperone system, and the mechanisms of steroid receptor signaling.

Conclusions

PP5 interacts with hsp90 through its TPR domain. The majority of PP5 is in the cytoplasm, consistent with its interaction with hsp90 *in vivo*. PP5 is a component of steroid receptor heterocomplexes containing hsp90. PP5 is present in both intermediate and mature steroid receptor heterocomplexes. Based on the use of a dominant negative PP5 mutant, PP5 may be important for glucocorticoid receptor signaling.

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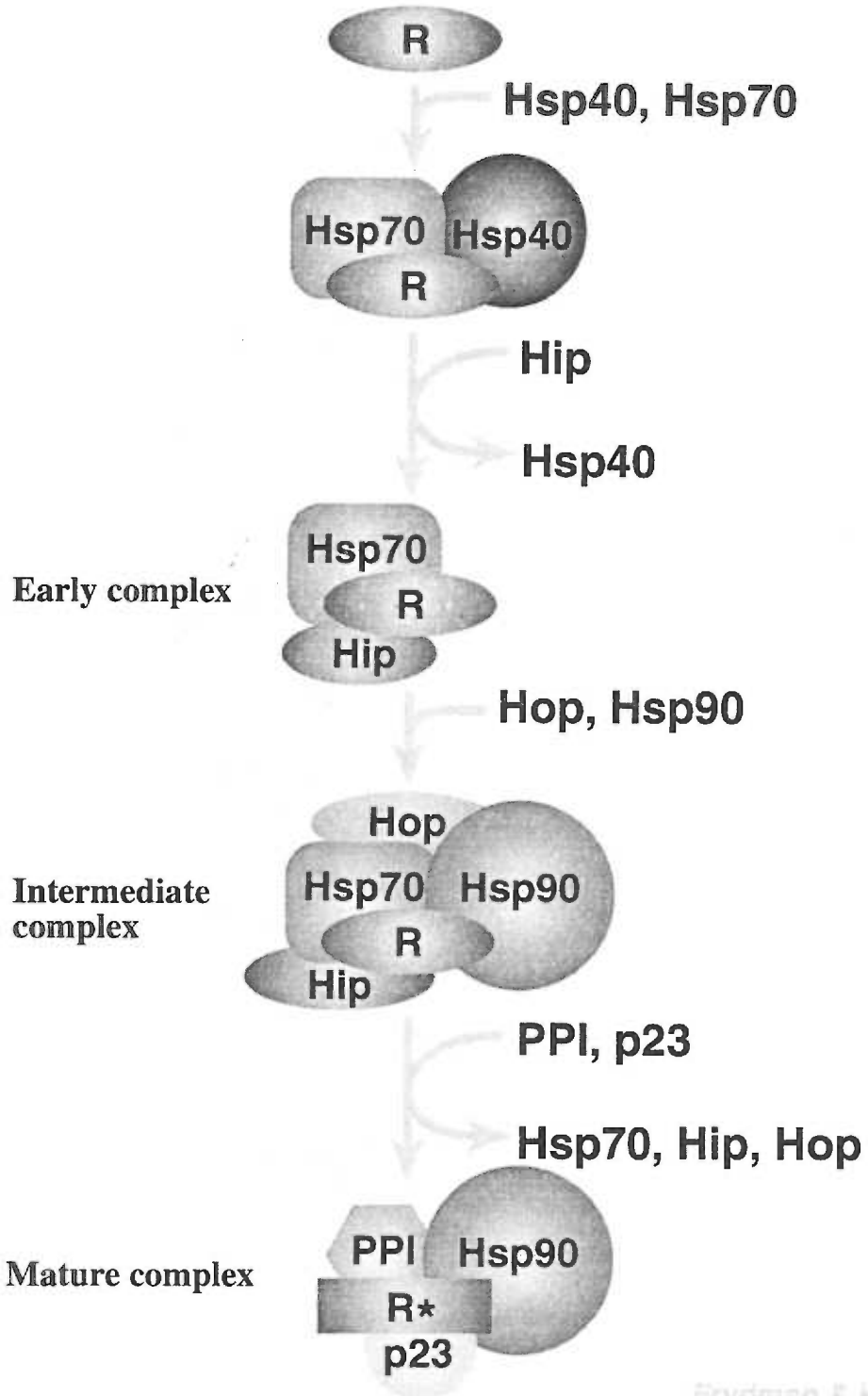
Fig. 1. A model for the steroid receptor heterocomplex assembly pathway



Frydman & Höhfeld
TIBS 22, 90 (1997)

Fig. 2. Geldanamycin Blocks the Transition between the Intermediate and Mature Heterocomplexes

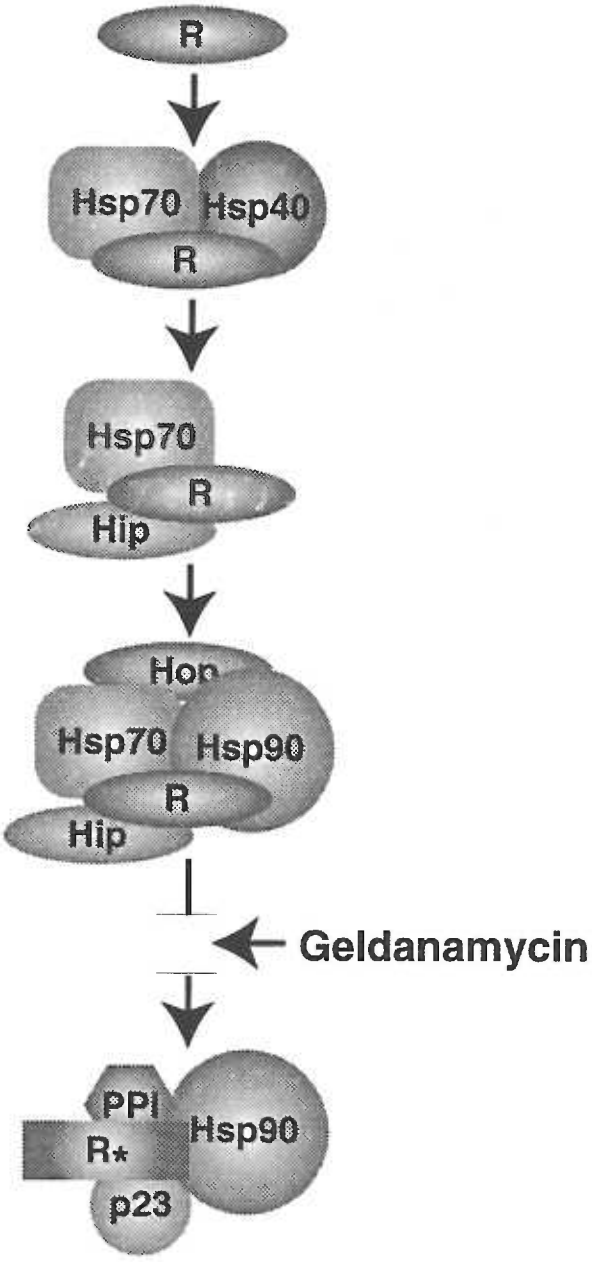


Fig. 3. Hsp90 co-immunoprecipitates with the FLAG-tagged TPR domain of PP5 or with FLAG-tagged PP5

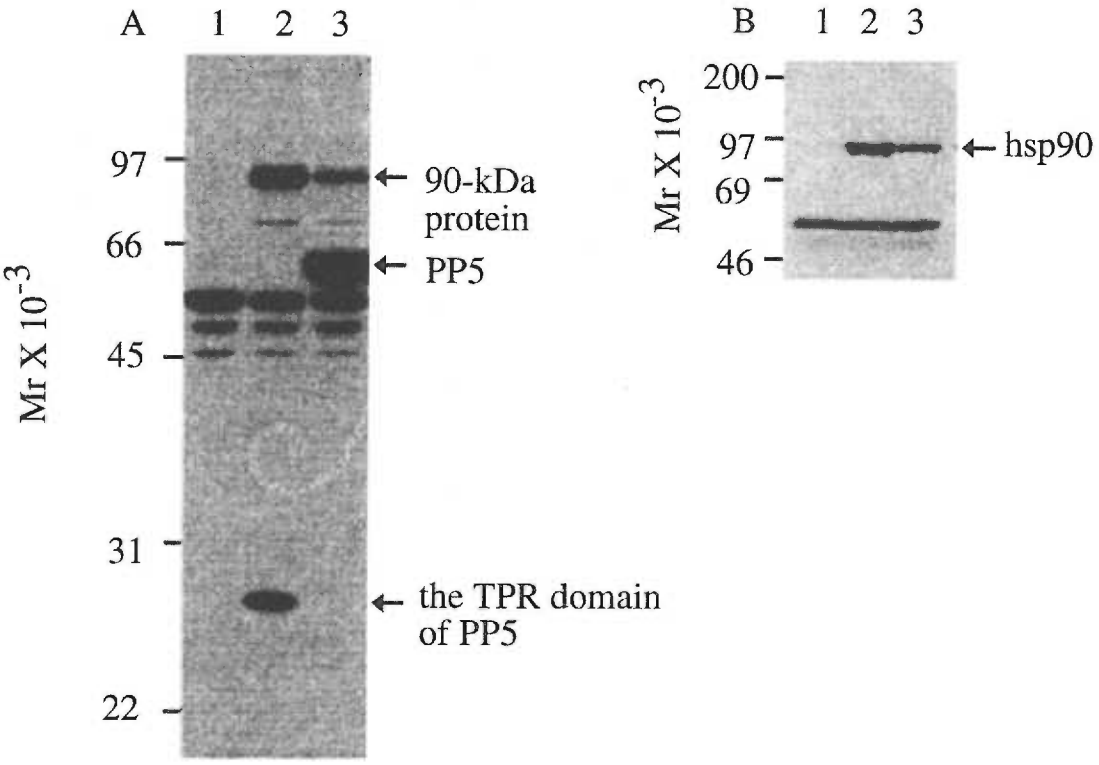


Fig. 3. Hsp90 co-immunoprecipitates with the overexpressed FLAG-tagged TPR domain of PP5 or with full-length FLAG-tagged PP5

COS-7 cells were transfected with pCMV6 as control plasmid (lane 1), with pCMV6-FLAG-TPR/PP5 (lane 2), or with pCMV6-FLAG-PP5 (lane 3) by a DEAE-dextran method. A, Cells were then labeled with [^{35}S]methionine/cysteine, and cell extracts were subjected to immunoprecipitation with a monoclonal antibody to the FLAG epitope. Immunoprecipitated proteins were analyzed by SDS-PAGE on a 12% gel, followed by fluorography. B, Immunoblotting of immunoprecipitates (prepared as in A but from unlabeled cell extracts and analyzed by SDS-PAGE on a 10% mini-gel) was performed with a monoclonal antibody to hsp90, and detected by chemiluminescence.

Fig. 4. SDS-PAGE of purified FLAG-tagged PP5

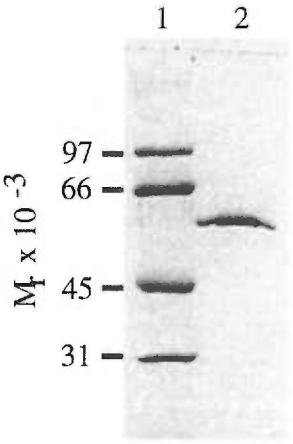


Fig. 4. SDS-PAGE of purified FLAG-tagged PP5

FLAG-tagged PP5 was purified from Sf9 cells as described in Material and Methods, and analyzed by SDS-PAGE and Coomassie Blue staining. Positions of molecular weight markers are indicated.

Fig. 5. Hsp90 co-immunoprecipitates with endogenous PP5 from COS-7 cells or rat brain

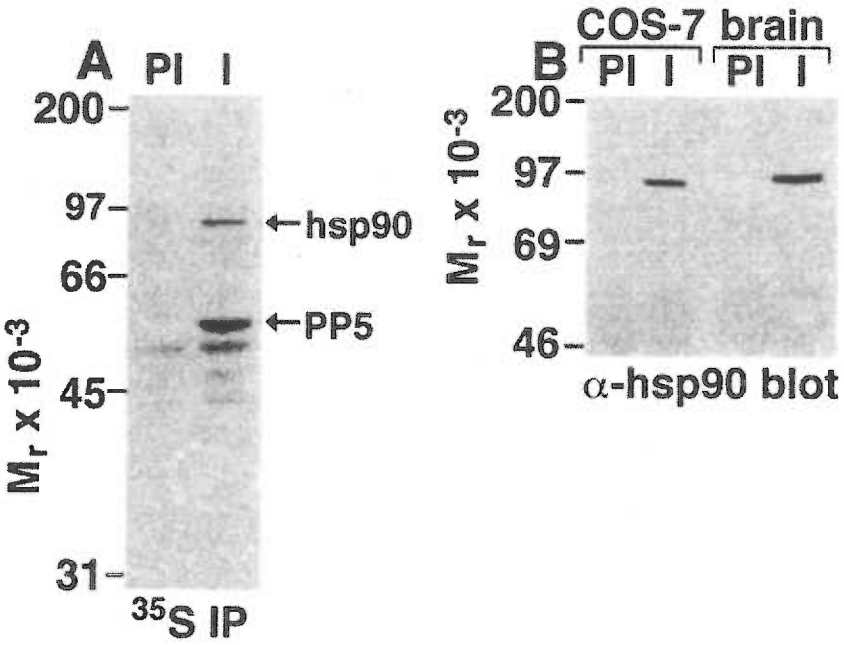


Fig. 5. Hsp90 co-immunoprecipitates with endogenous PP5 from COS-7 cells or rat brain

A, Extracts from untransfected COS-7 cells metabolically labeled with [^{35}S]methionine/cysteine were immunoprecipitated with preimmune serum (PI) or anti-PP5 serum (I). Immunoprecipitates were analyzed by SDS-PAGE on an 8.5% gel, followed by fluorography. B, Immunoprecipitates (prepared as in A but from extracts of unlabeled COS-7 cells or rat brain) were analyzed by SDS-PAGE on a 10% mini-gel, followed by immunoblotting with a monoclonal antibody to hsp90 and detection by chemiluminescence.

Fig. 6. Endogenous PP5 co-immunoprecipitates with endogenous hsp90

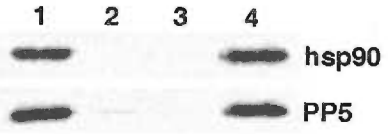


Fig. 6. Endogenous PP5 co-immunoprecipitates with endogenous hsp90

Aliquots of L929 cell cytosol were immunoprecipitated with nonimmune IgM or with the 3G3 monoclonal IgM against hsp90. Immunoblotting was performed using monoclonal antibody AC-88 to hsp90 or anti-PP5 serum. Immunoreactive bands were visualized using ^{125}I-conjugated secondary antibody and autoradiography. Lane 1, supernatant from nonimmune IgM adsorption; lane 2, supernatant from 3G3 antibody adsorption; lane 3, nonimmune pellet; lane 4, immune pellet.

Fig. 7. The majority of PP5 is in the cytoplasmic fraction

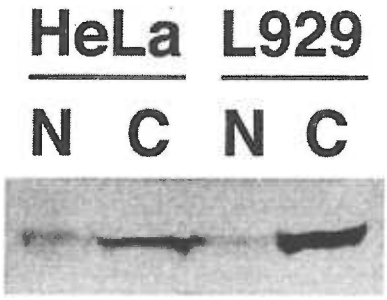


Fig. 7. The majority of PP5 is in the cytoplasmic fraction

Cytoplasmic and nuclear fractions of HeLa or L929 cells were analyzed by SDS-PAGE on an 8.5% gel. Immunoblotting was then performed using anti-PP5 serum, and visualized using peroxidase-conjugated secondary antibody and chemiluminescence. N: nuclear fraction, C: cytoplasmic fraction.

Fig. 8. Most PP5 is in the cytoplasm, as visualized by indirect immunofluorescence after paraformaldehyde fixation

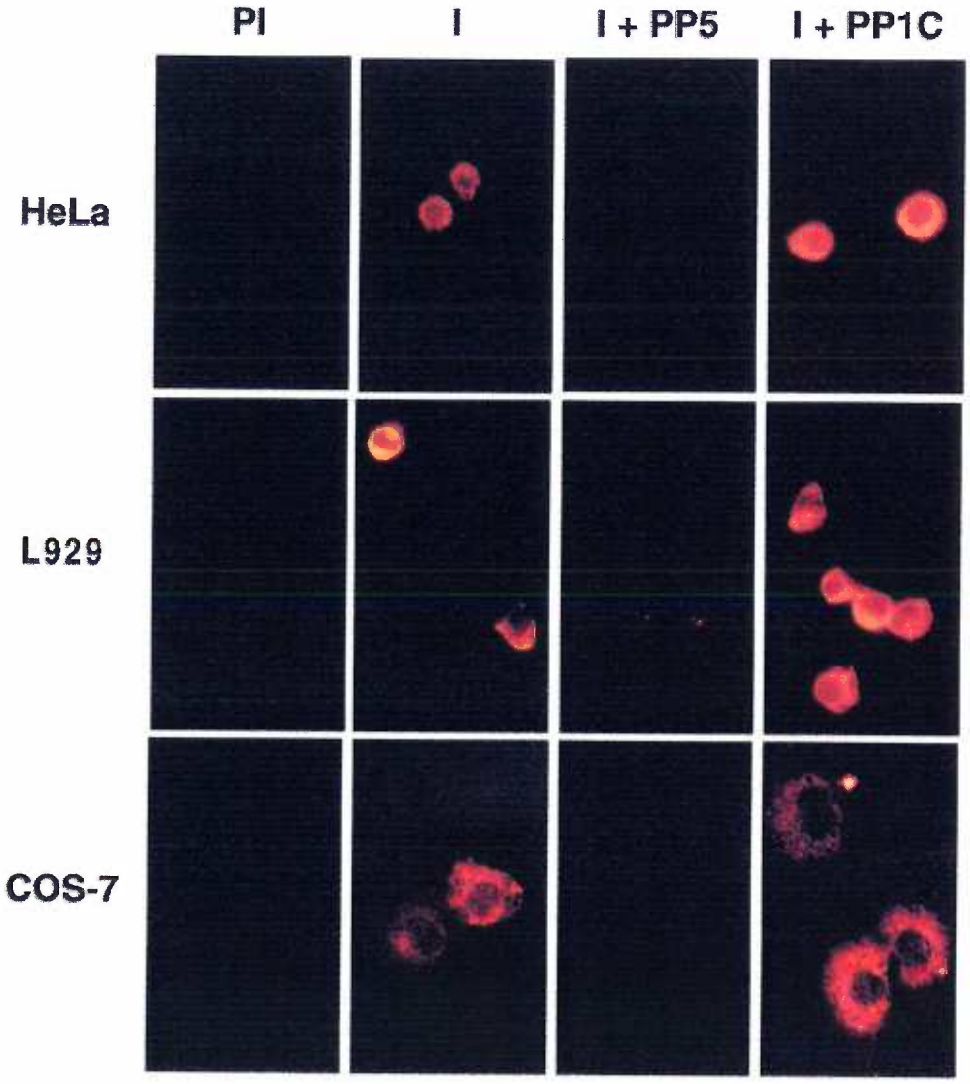


Fig. 8. Most PP5 is in the cytoplasm, as visualized by indirect immunofluorescence after paraformaldehyde fixation

HeLa, L929, and COS-7 cells were fixed with 2% paraformaldehyde and stained as described in Materials and Methods. Immunostaining was performed using the rhodamine-conjugated secondary antibody and visualized by a fluorescence microscope. The purified IgG from preimmune serum (PI) or anti-PP5 serum (I) was used as primary antibody. In order to show the specificity of anti-PP5 IgG for immunofluorescence, anti-PP5 IgG was preadsorbed with purified FLAG-tagged PP5 (I+PP5) or with the purified catalytic subunit of protein phosphatase 1 (I+PP1C) at 4°C overnight before incubation with permeabilized cells.

Fig. 9. Most PP5 is in the cytoplasm, as visualized by indirect immunofluorescence after acetone fixation

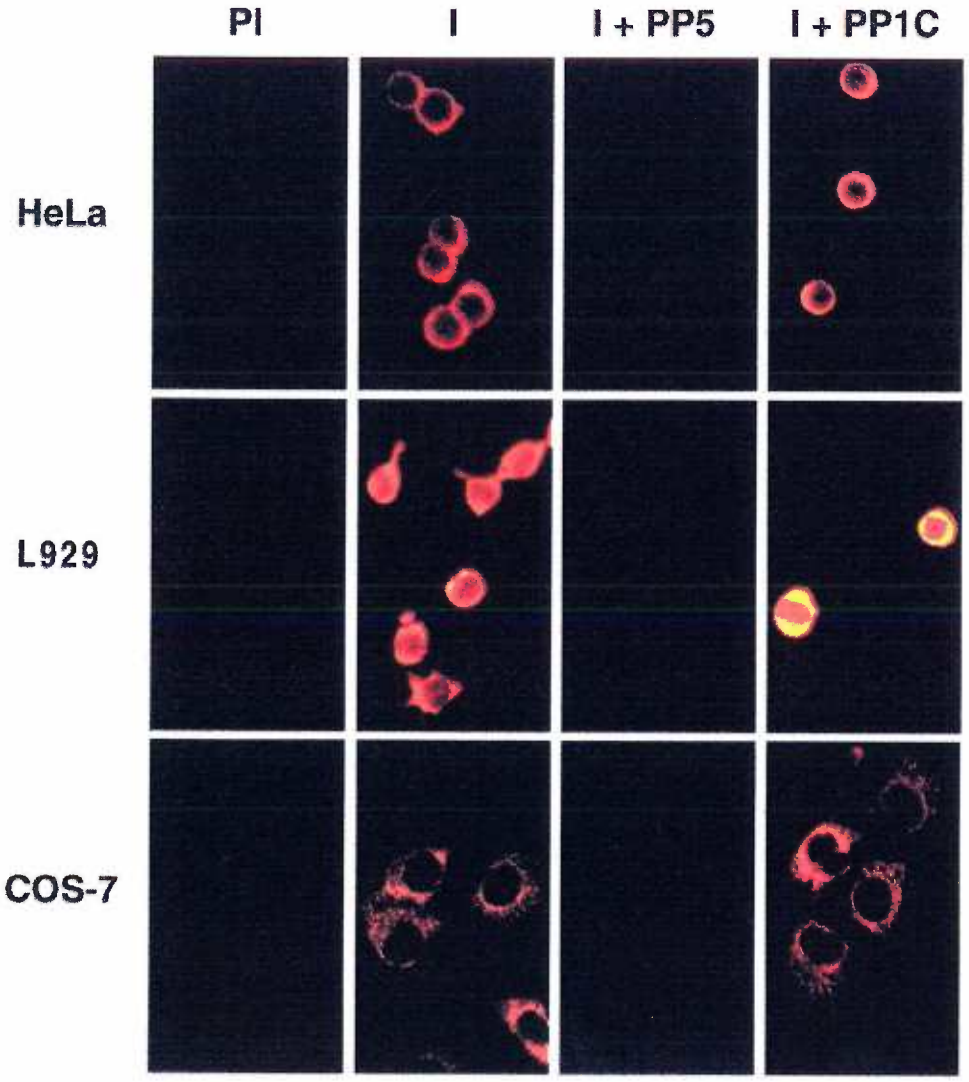


Fig. 9. Most PP5 is in the cytoplasm, as visualized by indirect immunofluorescence after acetone fixation

HeLa, L929, and COS-7 cells were fixed with acetone and stained as described in Materials and Methods. Immunostaining was visualized using a rhodamine-conjugated secondary antibody and a fluorescence microscope. The purified IgG from preimmune serum (PI) or anti-PP5 serum (I) was used as primary antibody. In order to show the specificity of anti-PP5 IgG for immunofluorescence staining, anti-PP5 IgG was preadsorbed with purified FLAG-tagged PP5 (I+PP5) or with the purified catalytic subunit of protein phosphatase 1 (I+PP1C) at 4˚C overnight before incubation with permeabilized cells.

Fig. 10. Hsp90 and PP5 are present in a high molecular weight complex

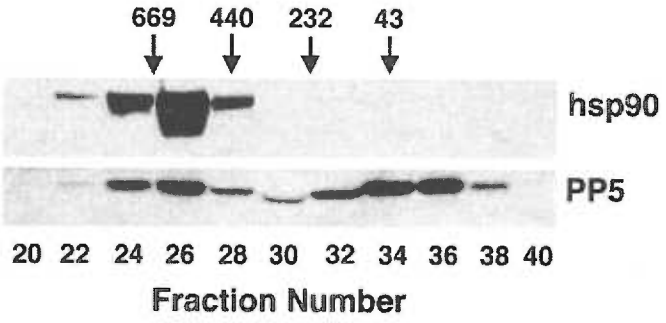


Fig. 10. Hsp90 and PP5 are present in a high molecular weight complex

Rat brain extract was fractionated on a Superose 6 column, and aliquots of fractions were analyzed by SDS-PAGE on an 8.5% gel. Immunoblotting was performed using the AC-16 antibody to hsp90 or anti-PP5 serum as indicated, and detected by chemiluminescence. The positions of protein markers used to calibrate the column are indicated by arrows. Molecular weights of marker proteins are shown in kilodaltons.

Fig. 11. PP5 co-immunoprecipitates with the glucocorticoid receptor

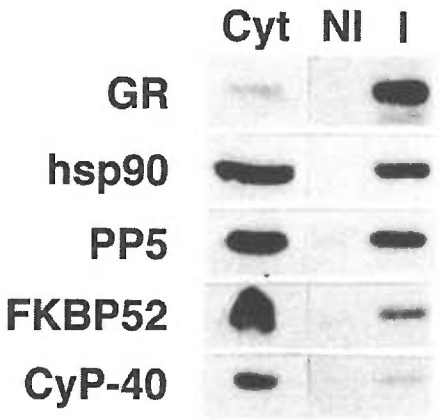


Fig. 11. PP5 co-immunoprecipitates with the glucocorticoid receptor

Aliquots of L929 cell extracts were immunoprecipitated with nonimmune mouse IgG (lane 2) or the BuGR2 monoclonal antibody against GR (lane 3). Immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with antibodies against the proteins indicated at the left. Immunoreactive bands were visualized using ^{125}I-conjugated secondary antibody and autoradiography. Lane 1, 20 µl of L929 cell extract.

Fig. 12. The TPR domain of PP5 acts as a dominant negative mutant to block GR-mediated transcriptional activation

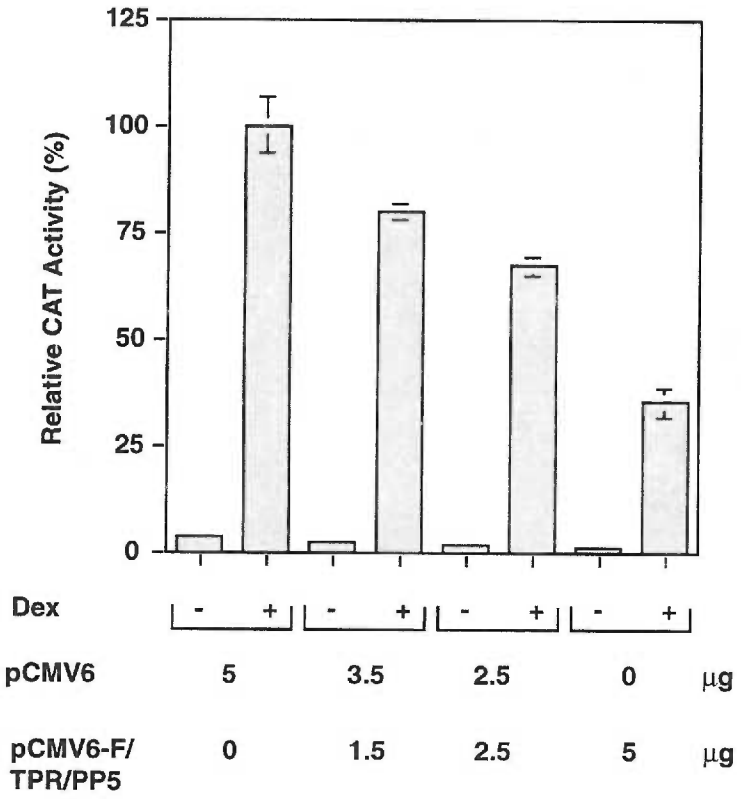


Fig. 12. The TPR domain of PP5 acts as a dominant negative mutant to block GR-mediated transactivation

CV-1 cells were co-transfected with plasmids encoding GR, a CAT reporter gene, and luciferase and the indicated amounts of pCMV6 and pCMV6-FLAG-TPR/PP5. Luciferase and CAT activity were measured after incubation with 100 nM dexamethasone or 0.1% DMSO vehicle at 37°C for 26 h. CAT activity was normalized to luciferase activity to control for variations in transfection efficiency. Data from two experiments, triplicate for each experiment, are expressed as the mean ± S.E.. Dexamethasone-induced CAT activity in the absence of pCMV6-FLAG-TPR/PP5 is defined as 100%.

Fig. 13A. Expression of the TPR domains of PP5, Hop, and FKBP52 in COS-7 cells

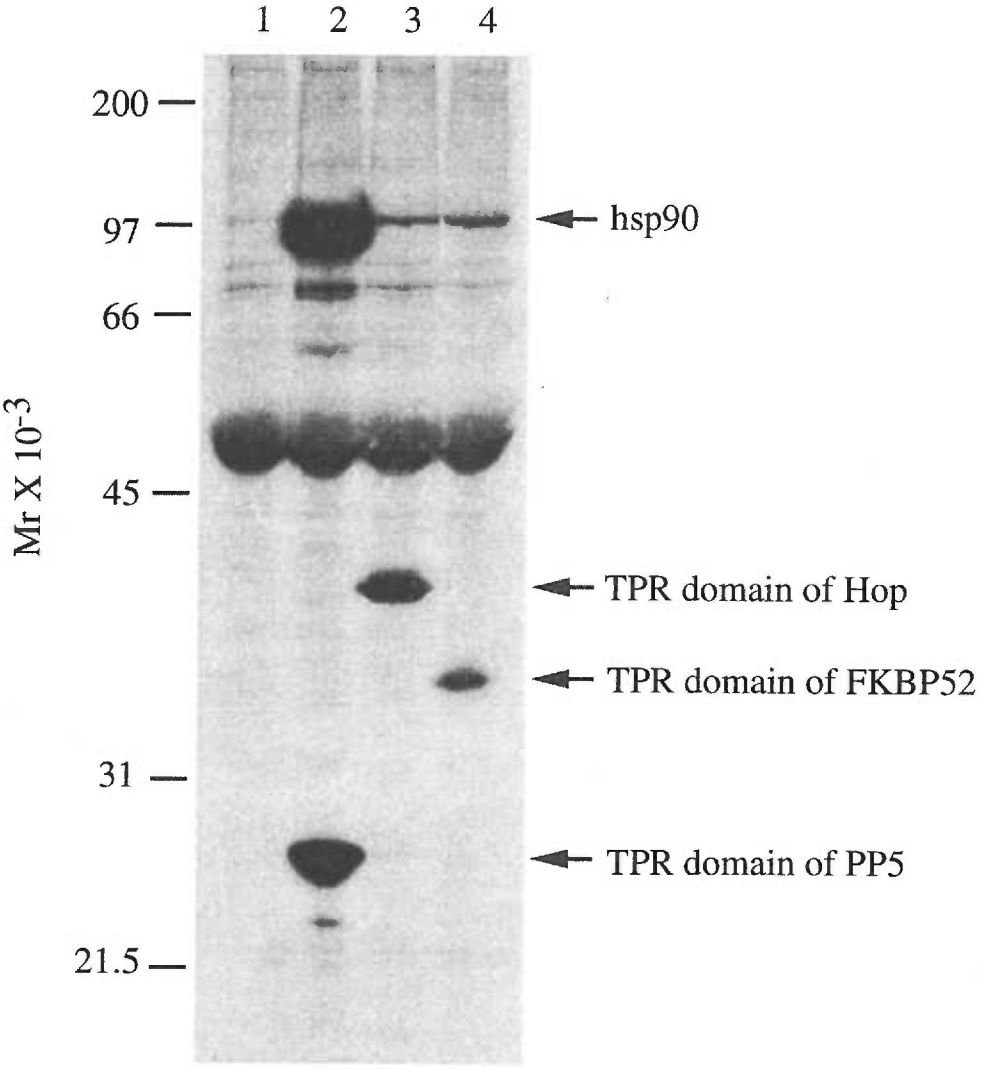


Fig. 13B. Effects of the TPR domains of PP5, Hop, and FKBP52 on GR-mediated transactivation

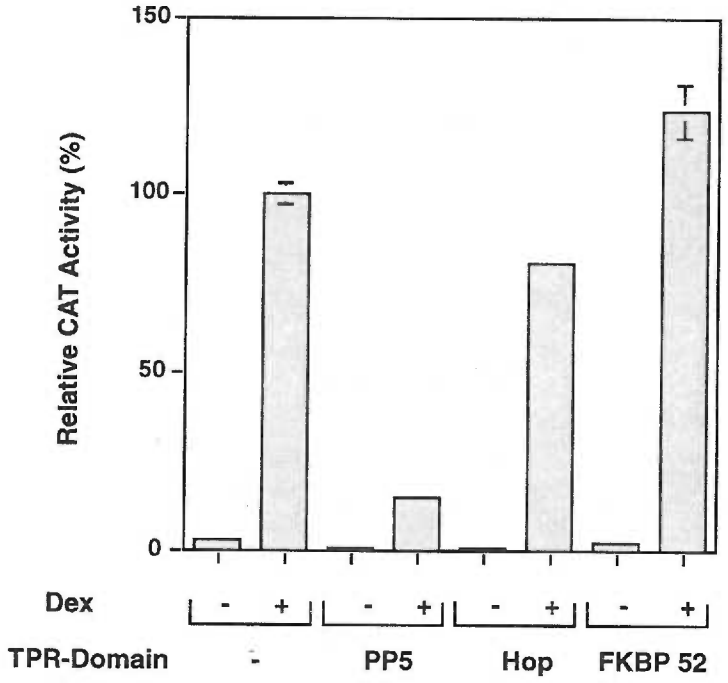


Fig. 13. Effects of the TPR domains of PP5, Hop, and FKBP52 on GR-mediated transactivation

A, Expression of the TPR domains of PP5, Hop, and FKBP52 in COS-7 cells. COS-7 cells were transfected with pCMV6 (lane 1), pCMV6-FLAG-TPR/PP5 (lane 2), pCMV6-FLAG-TPR/Hop (lane 3), pCMV6-FLAG-TPR/FKBP52 (lane 4). Cells were then labeled with [^{35}S]methionine/cysteine, and cell extracts were subjected to immunoprecipitation with antibody to the FLAG epitope. Immunoprecipitated proteins were analyzed by SDS-PAGE on a 12% gel, followed by fluorography. B, Effects of the TPR domains of PP5, Hop, and FKBP52 on GR-mediated transactivation. CV-1 cells were co-transfected with plasmids expressing GR, a CAT reporter gene, and luciferase, and 5 μg of pCMV6, pCMV6-FLAG-TPR/PP5, pCMV6-FLAG-TPR/Hop, or pCMV6-FLAG-TPR/FKBP52. Luciferase and CAT activities were measured after incubation with 100 nM dexamethasone or 0.1% DMSO vehicle at 37°C for 26 h. CAT activity was normalized to luciferase activity to control for variations in transfection efficiency. Data from two experiments, triplicate for each experiment, are expressed as the mean ± S.E.. Dexamethasone-induced CAT activity in the presence of pCMV6 is defined as 100%.

Fig. 14. PP5 is in the heterocomplex containing Hop, and the heterocomplex containing p23

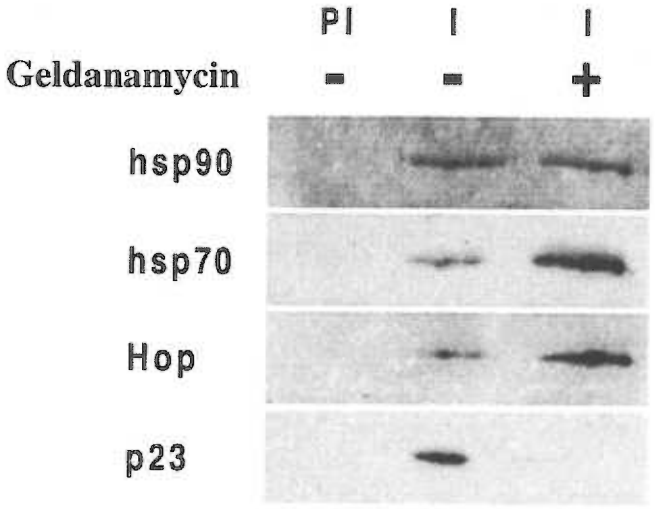


Fig. 14. PP5 is in the heterocomplex containing Hop, and the heterocomplex containing p23

L929 cell extracts made from cells treated with 1 μM geldanamycin (+) or 0.1% DMSO vehicle (-) at 37°C for 18 h were immunoprecipitated with preimmune serum (PI) or anti-PP5 serum (I). Immunoprecipitates were analyzed by SDS-PAGE followed by immunoblottings with antibodies against the proteins indicated at the left. Immunoreactive bands were visualized by chemiluminescence.