Differential regulation of glucocorticoid and progesterone receptor subcellular localization by tetratricopeptide repeat domain proteins

Ananya Banerjee
Medical University of Ohio

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**FINAL APPROVAL OF DISSERTATION**

*Doctor of Philosophy in Biomedical Sciences*

Differential Regulation of Glucocorticoid and Progesterone Receptor Subcellular Localization by Tetratricopeptide Repeat Domain Proteins

Submitted by:
Ananya Banerjee

In partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences

**Examination Committee**

**Major Advisor:** Edwin R. Sanchez, Ph.D.

**Academic Advisory Committee:**
- William Maltese, Ph.D.
- Linda Dokas, Ph.D.
- Ronald Mellgren, Ph.D.
- Han-Fei Ding, Ph.D.

**Senior Associate Dean**
College of Graduate Studies
Michael S. Bisesi, Ph.D.

**Date of Defense:** March 22, 2007
Differential Regulation of Glucocorticoid and Progesterone Receptor Subcellular Localization by Tetra-tricopeptide Repeat Domain Proteins

Ananya Banerjee

University of Toledo
2007
DEDICATION

The two individuals who have made every bit of this long and arduous journey absolutely worthwhile are my mother and father. Their unconditional support and constant motivation gave me the courage to tide over difficult times and accept new challenges that crossed my path. I thus dedicate this endeavor to them.
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INTRODUCTION

Steroid receptors under unliganded conditions exist in hetero-oligomeric complexes containing several molecular chaperones. During the course of assembly, steroid receptors interact with several chaperone components that are not present in the mature heterocomplex (Smith, 1993; Pratt and Toft, 1997). In the steady state, most complexes contain the heat shock protein 90 (HSP90) dimer, which in turn recruits HSP90-binding protein p23, and one of the four major tetratricopeptide repeat (TPR) proteins. Only the larger members of the FK506 and cyclosporineA (CsA) binding families of immunophilins, such as FK506-binding protein 51 (FKBP51), FK506-binding protein 52 (FKBP52) and cyclophilin-40 (Cyp40), bind competitively via their TPR domains to a single site generated at the C-terminus of the HSP90 dimer. Thus, HSP90-bound steroid receptor complexes are individually distinguished by the immunophilin content. Besides being ubiquitously expressed, immunophilins are highly conserved proteins that possess peptidyl prolyl cis-trans isomerase (PPIase) activity proposed to catalyze protein folding (Galat, 1993).

The binding of immunosuppressant drugs such as FK506, rapamycin and CsA have been shown to inhibit the PPIase activity suggesting the dual role of this domain in ligand-binding and catalysis (Schreiber and Crabtree, 1992). Protein phosphatase 5 (PP5) that binds FK506 with low affinity and shows some sequence homology with the FKBP PPIase domain actively competes with other immunophilins for the TPR acceptor site on HSP90. However, constant assembly and disassembly results in the presence of early and
intermediate complexes alongside functionally mature receptor conformations (Barent et al., 1998).

The continued presence of TPR proteins in mature steroid receptor complex has led to the general notion that, besides chaperoning, these proteins also could be involved in regulation of receptor function (Cheung-Flynn and Smith, 2000). Although relative abundance and HSP90-binding affinity might be critical in determining immunophilin incorporation into steroid receptor complexes, the possibility that each of these proteins might harbor specificity for individual receptors is supported by numerous observations. In cell-free assembly systems, progesterone receptor (PR) shows a preferential association with FKBP51, in spite of an existing five-fold difference between the levels of FKBP51 and FKBP52 (Nair et al., 1997). However, in *in vitro* systems, although PR has been shown to bind FKBP51, no recruitment of PP5 has been reported so far (Barent et al., 1998). Our findings are in accordance with published reports, and in the cell types used in this study we were able to identify the preferential association of PR with FKBP52 and FKBP51. The PR heterecomplexes in our hands showed no interaction with PP5 confirming previous reports.

A biochemical investigation revealed differences in the binding affinities of the TPR proteins for HSP90, with FKBP52 displaying the highest affinity followed by FKBP51 and finally Cyp40 (Pirkl and Buchner, 2001). However, comparison of the chaperone function of these proteins *in vitro*, revealed that Cyp40 and FKBP51 functioned as more potent chaperones than FKBP52 (Pirkl and Buchner, 2001). Surprisingly, in the case of both FKBP52 and Cyp40, chaperoning function has been
demonstrated to be independent of PPIase function, although FKBP52 does show the requirement of an intact TPR domain (Bose et al., 1996; Freeman et al., 1996; Pirkl et al., 2001).

The first definitive evidence showing the involvement of TPR immunophilins in modulating receptor hormone responsiveness came from studies which showed that elevated expression of FKBP51 over FKBP52 led to its increased incorporation into the receptor heterocomplex, thereby lowering the hormone-binding affinity of the receptor (Reynolds et al., 1999). Further, by using squirrel monkey lymphoblasts (SML), interaction with HSP90 was identified as one of the critical requirements for the inhibitory effect of FKBP51 on glucocorticoid receptor (GR) while PPIase activity had no contribution (Denny et al., 2005). The inhibitory effect of FKBP51 on GR hormone-binding was more pronounced with squirrel monkey FKBP51 compared to humans (Denny et al., 2000). The differences between the N and C terminal amino-acid sequences of squirrel monkey and human FKBP51 may contribute towards such an effect by impeding the interaction of this protein with the receptor or restricting the conformational changes that normally occur in the receptor following ligand-binding (Ratajczak et al., 2003).

Elevated levels of FKBP51 were reported to increase androgen receptor (AR) transcriptional activity (Febbo et al., 2005). This is in contrast to previous findings demonstrating FKBP51 to be a negative regulator of GR and PR transcriptional activity (Denny et al., 2000; Hubler et al., 2003). “Hormone-induced” expression of FKBP51 and its role in attenuating the hormone-response in conditioned tissues clearly demonstrates
the existence of an autoregulatory loop. Thus, distinct interactions between different steroid receptors and immunophilins via HSP90 can be summarized to have both stimulatory and inhibitory effects on receptor activities. The function that is likely to be directly affected by inhibition of the receptor’s hormone-binding capacity would be receptor translocation and this could directly influence subcellular localization.

The contribution of TPR protein FKBP52 towards receptor function as well as localization is somewhat better researched. The “hormone-induced” switch from immunophilin FKBP51 to FKBP52 within the cytosolic GR heterocomplex has been shown to cause nuclear localization of the heterocomplex (Davies et al., 2002). The unique ability of FKBP52 to interact with HSP90 in the steroid receptor complex and simultaneously bind dynein at the PPIase domain allows it to perform the function of an adaptor molecule facilitating GR transport along the cytoskeletal tracts (Galigniana et al., 1998; 2002). The role of FKBP52 in regulating receptor function became apparent when over-expressing hFKBP52 in yeast led to an increase in GR transcriptional response (Riggs et al., 2003). However, similar investigations in the mammalian system questioned the validity of the previous observations in yeast. Over-expression of FKBP52 had no effect on GR activity, although increased expression of FKBP51 did decrease the transcriptional response (Wochnik et al., 2005). Taken together, these findings suggest that the hormone binding function of GR appears to be tightly regulated by the TPR component of the complex, with FKBP52 playing the stimulatory partner and targeting the receptor complex to the nucleus, while FKBP51 leading to cytoplasmic retention of
the receptor by virtue of its inhibitory role (Denny et al., 2000; Cheung-Flynn et al., 2003).

Recent reports not only show an indirect association between the PPIase domain of Cyp40 and the dynamitin component of the dynein complex but also reveal localization of Cyp40 along the microtubules (Galigniana et al., 2004b). The contribution of PP5 to GR functionality is far from being clear. Under *in vitro* conditions, in the presence of immunosuppressant drugs such as FK506, exchange of FKBP51 for PP5 within the GR complex is consistent with an increase in receptor’s hormone-binding and decrease in the total number of hormone-binding sites (βmax) (Davies et al., 2005). Suppression of PP5 using antisense against PP5 (ISIS15534) in A549 cells resulted in nuclear accumulation of GR with no apparent effect on hormone-binding, highlighting the fact that aberrant PP5 expression may be implicated in the development of human cancers by inhibiting the GR-induced antiproliferative cascade and promoting cell proliferation (Zuo et al., 1999).

Despite similarities in the nuclear localization signal (NLS) sequences and a common association with heat shock proteins, steroid receptors are differentially localized in the cell in the absence of ligand. Unoccupied PR was predominantly localized in the nucleus of luminal and glandular epithelium cells of the uterus, (Perrot-Applanat et al., 1985) yet unliganded GR localized to the cytoplasmic compartment of the same cells (Wikstrom et al., 1987). While several features in the N-terminal half of the receptor’s ligand binding domain (LBD) are critical determinants of localization, (Wan et al., 2001) the role of TPR proteins in this process remains to be elucidated. The
best-characterized functions of these molecular chaperones are their roles in assembly and stabilization of steroid receptor heterocomplexes. Thus based on the above-mentioned observations, it appears that cellular distribution of steroid receptors is a complex process involving more than one player. Besides the nuclear localization signals directly encoded in the receptor, immunophilin composition of the large macromolecular complexes formed might have a major contributory role. I propose that subcellular localization of steroid receptors in a particular cell line is largely determined by the receptor’s TPR specificity but may be influenced by cell-type-specific TPR expression pattern. This work attempts to integrate the existing knowledge of factors regulating receptor localization as well as provide evidence in favor of TPR involvement in this process.

**Significance of manuscript**

Little is known about the mechanisms that facilitate steroid receptor subcellular localization and the potential role of TPR proteins in these processes. The overall aim of this work is to provide clearer insight into how TPR proteins actually influence receptor localization. We begin by showing that differential localization of GR in L929 and WCL2 cells can be attributed to their differences in specificity towards TPR proteins. In WCL2 cells, higher equilibrium expression levels of FKBP52 compared to FKBP51 might be causal in the recruitment of this TPR into GR complexes. In L929 cells, as in most other reported cell-types and tissues (Wikstrom et al., 1987), GR showed a
cytoplasmic distribution and was mostly bound to FKBP51, which has the highest expression level amongst all other TPR proteins.

In L929 cells, immunoadsorption of FKBP52 component of steroid receptor-HSP90 heterocomplexes was accompanied by co-adsorption of cytoplasmic motor protein dynein (Galigniana et al., 2001). Also in NIH 3T3 cells dexamethasone-dependent translocation of GFP-GR from the cytoplasm to nucleus was inhibited by co-transfection of FKBP52 PPIase domain but not by FKBP12 PPIase domain (Galigniana et al., 2001). Further studies showed that treatment of these cells with colcemid to eliminate microtubules prior to steroid addition resulted in the loss of the inhibitory effect of the PPIase domain of FKBP52 on receptor translocation and this effect was restored on withdrawal of colcemid. Moreover, the cellular distribution of FKBP52 in most cell types, such as the WCL2 and HEK cells is predominantly cytoplasmic and along the microtubules (Ruff et al., 1992; Czar et al., 1994). These observations suggest that by interacting with dynein at its PPIase domain, FKBP52 targets retrograde transport of GR along the microtubules by functioning as an adaptor between receptor and dynein motor complex (Galigniana et al., 2002; Harrell et al., 2004). Although literature supports an interaction between the PPIase domain of Cyp40 (Galigniana et al., 2004b) and dynein, we ruled out this possibility since GR complexes in both cell types showed no interaction with Cyp40 under hormone-free conditions. PP5 is capable of binding dynein (Galigniana et al., 2002) and with its almost exclusively cytoplasmic distribution in many cell types (Borthwick et al., 2001) including L929 and WCL2 cells, makes it well poised to be a contender involved in nuclear transport of GR.
FKBP51 purportedly lacks the ability to interact with dynein (Wochnik et al., 2005) and shows a fairly uniform distribution between the cytoplasmic and nuclear compartments. Based on these observations, it would be reasonable to speculate on the involvement of FKBP51 in either redistribution or retention of GR to the cytoplasm. Another interesting observation that we went onto reconfirm was the fact that, although unliganded GR in WCL2 cells was predominantly nuclear, it was not tightly bound to this compartment and could be released into the cytosolic fraction upon cell rupture. Treatment with dexamethasone led to recovery of most of the cellular GR in the nuclear fraction demonstrating that, in the presence of ligand, the receptor was converted to a high affinity nuclear binding form (Sanchez, 1992; Shen et al., 1993). The unliganded mouse GR over-expressed in CHO-derived WCL2 cells was not just nuclear but also showed a consistent association with both HSP70 and HSP90, while the receptor in L929 cells only bound HSP90 (Sanchez et al., 1990). It is unlikely that this observation could account for the nuclear localization of unliganded GR in these cells, since CHO cells selected for an intermediate levels of receptor expression comparable to that of L929 cells (CHO-W3) also were bound to HSP70 (Sanchez et al., 1990).

After having identified the TPR proteins responsible for differential localization of GR in both cell types, we proceeded to determine if localization of GR in these cell types could be affected by altering the equilibrium levels of FKBP51 and FKBP52. We achieved this by expressing FLAG-tagged constructs of FKBP51 and FKBP52. In WCL2 cells, expression of FLAG-tagged FKBP51 led to the redistribution of GR to the cytoplasm following its recruitment into the heterocomplexes. In the case of L929 cells
localization of unliganded GR remained unaffected since no FLAG52 entered the heterocomplexes and neither was there any increase in the amounts of endogenous FKBP52 being recruited to the complexes. Our findings suggest that the receptor’s intrinsic affinity for FKBP51 is the key element in promoting its cytoplasmic localization. However, in WCL2 cells, the affinity of GR for FKBP51 perhaps could be out competed by FKBP52 in a manner that is not completely determined by its relative abundance.

The ability of PR complexes to bind FKBP51 or FKBP52 (Tai et al., 1986; Smith et al., 1990; Barent et al., 1998) along with GR’s specificity for FKBP51 and PP5 have been well documented (Chen et al., 1996; Davies et al., 2002) but the contribution of these proteins towards determining steroid receptor subcellular localization has barely been addressed. Having examined the TPR control of GR localization, we wanted to investigate if localization of other steroid receptors was regulated by their TPR preferences as well. Co-IP assays performed in receptor-less COS-1 cells expressing green fluorescent protein (GFP)-tagged GR or PR-B, revealed differences in TPR recruitment to be instrumental in the differential localization observed by GFP fluorescence.

Under untransformed conditions, localization of both receptor subtypes correlated well with their TPR preferences. PR-B complexes mostly bound FKBP52 and were primarily localized in the nucleus with a small cytoplasmic receptor population, as reported previously (Lim et al., 1999; Wan et al., 2001). The GR complexes, which mainly recruited FKBP51, remained predominantly cytoplasmic under hormone-free
conditions. Similar studies performed using chimeric constructs of GR and PR-B, helped to identify the LBD of the receptors as the site that controlled localization as well as TPR specificity. In light of these findings, the receptor’s regulation of TPR specificity and in turn its localization via the signal transduction region of the LBD localization is now much less of a conundrum.

Finally, to assess the active role of each of the TPR proteins in receptor localization we decided to compare the localization of GR and PR-B in wild-type (WT) mouse embryonic fibroblasts (MEF) to a series of knock out (KO) MEF cell lines each missing a specific TPR protein. Unliganded GR in FKBP51 KO MEFs displayed a greater nuclear distribution compared to its predominant cytoplasmic distribution in the WT MEFs. This observation supports the proposed role of FKBP51 in cytoplasmic retention or redistribution of the receptors. The localization of unliganded PR-B in FKBP51 KO MEFs resembled that of PR-A (Lim et al., 1999) with the entire receptor population including the small cytoplasmic fraction of PR-B now distributed in the nucleus. Localization of unliganded GR in FKBP52 KO cells remained unaffected confirming our findings in L929, WT MEF and COS-1 cells where we noted a lack of association between GR complexes and FKBP52. A dramatic change in the distribution of untransformed PR-B with almost the entire receptor population becoming cytoplasmic reconfirmed the preference of this receptor for FKBP52. We speculate that in the absence of FKBP52, TPR protein FKBP51 may be actively involved in redistribution of the receptor to the cytoplasm.
Localization of GR in PP5 KO cells was predominantly cytoplasmic and comparable to that in the WT MEFs suggesting the possibility that this TPR may not have an indispensable role with respect to localization. Although it is worth mentioning the fact that loss of PP5 appears to be completely compensated by other TPR proteins, possibly FKBP51 since GR distribution remained unaffected in this KO cell line. However the reverse case did not hold true since untransformed GR in FKBP51 KO cells showed a greater nuclear localization. Lack of association with PP5 both in our findings as well as published reports accounts for unaltered localization of mature PR-B in these cell. Lastly, the unaffected receptor translocation in the three KO MEF cell lines is best explained by the apparent redundancy in the ability of FKBP52, PP5 and even Cyp40 to direct receptor complexes to dynein.

Nordeen and colleagues have demonstrated using very elegantly designed experiments that the LBD domain of unoccupied steroid receptors, such as GR and PR determines their equilibrium distribution (Wan et al., 2001). Taking this investigation to the next level, we not only reconfirmed the role of the LBD in controlling localization but also identified its previously unrecognized contribution towards controlling the receptor’s TPR specificity. Future studies will help to reveal if TPR selectivity by receptors occurs at the level of HSP90 or whether distinct conformations of helices 1-5 of the LBD of the steroid receptor establish the identity of the TPR being recruited.
LITERATURE

Steroid receptors behave as ligand-dependent transcription factors regulating the expression of target genes in response to specific agonists or antagonists (Jensen, 1996). Transcriptional regulation occurs through recruitment of receptor coactivators, corepressors and chromatin-modifying enzymes (Shibata et al., 1997). Conditional interaction of NLS with the import/export apparatus in the nuclear pore has often been used as one of the criteria for determining cytoplasmic versus nuclear distribution of steroid receptors (Tyagi et al., 1998). The structure of TPR proteins, their relative abundance, binding affinity for HSP90 and, most importantly, their ability to interact with specific steroid receptors could potentially influence receptor preferences. The following sections discusses the varied functional consequences resulting from the association of steroid receptors with different immunophilins.

STEROID RECEPTOR STRUCTURE

FIGURE 1. DOMAIN ORGANIZATION OF NUCLEAR RECEPTORS

Nuclear hormone receptors display a modular, highly conserved structure with six major regions. The C-terminal region encompasses the ligand-binding domain (LBD), which is well conserved among nuclear receptors (Figure 1). It contains twelve alpha helices of which helices H9 and H10 form the dimerization interface. The functional
integrity of the ligand-dependent transactivation function (AF-2) present in this domain relies upon the highly conserved amphipathic helix H12 located at the C-terminal end of the LBD.

The LBD is structured into a three-layered anti-parallel helical sandwich with the central core layer creating the ligand-binding pocket. This region has hydrophobic residues and is buried within the bottom half of the LBD. Conformational changes induced by ligand-binding lead to the creation of a new surface, which permits coactivators to be recruited (Glass and Rosenfeld, 2000). These coactivators often possess intrinsic acetyl transferase activity allowing them to recruit other large complexes for histone remodeling (Narlikar et al., 2002) such as histone acetyltransferase (PCAF) (Roth et al., 2001), histone methyltransferase (CARM1/PRMT1) (Kouzarides, 2002) and histone kinase (Merienne et al., 2001). Decondensing chromatin via histone remodeling activities creates a transcriptionally active environment.

The N-terminal region shows the greatest variability in length and sequence. The transcription activation domain (AF-1) present in this region acts autonomously and ligand independently of ligand (Nagpal et al., 1993). The most noteworthy feature of the AF-1 domain is the presence of several consensus phosphorylation sites, which make this region an ideal target for multiple kinases. Some recent reports have shown a synergistic interaction between AF-1 and AF-2 facilitated by the recruitment of coactivators to each of these domains (Benecke et al., 2000).

The central DNA-binding domain (DBD) is the most conserved of all regions containing two alpha helices along with two type-II zinc finger regions that are involved
in recognition of cognate response elements (Gelman et al., 1999; Benecke et al., 2000). The DBD contains nine cysteines, along with some other residues that are conserved across the nuclear receptor super family. The P box present at the base of the first zinc finger contains amino acid residues required for discrimination of core DNA recognition motifs. The residues of the second zinc finger form the D box involved in receptor dimerization (Aranda and Pascual, 2001). The majority of the residues that are phosphorylated in nuclear receptors lie within the variable N-terminal half. They are usually serine residues surrounded by prolines, consequently serving as consensus sites for cyclin-dependent kinases (CDKs) (Morgan, 1995) and mitogen-activated protein kinases (MAPKs) (Pearson et al., 2001). Many of these sites may be phosphorylated by CDKs in the absence of hormone or under liganded conditions (Weigel, 1996).

The hinge region following the DBD is not well conserved among different steroid receptors and, besides serving to link the DBD to the LBD, it permits rotation of the DBD. Along with the nuclear localization signal (NL1), this region contains residues crucial for interaction with corepressors. Steroid receptors bind almost exclusively after homodimerizing to response elements arranged as inverted (palindromic) repeats of two consensus hexameric half sites separated by three spacer nucleotides (Green et al., 1988).

**STRUCTURE AND FUNCTION OF THE GLUCOCORTICOID RECEPTOR**

Natural glucocorticoids as well as their synthetic derivatives work through the GR, a member of the nuclear hormone receptor super family of ligand-activated transcription factors. The levels of circulating glucocorticoids are regulated by
adrenocorticotropic hormone (ACTH) largely under the control of the hypothalamic pituitary adrenal axis (HPA). Most of the metabolic effects of glucocorticoids result from increased transcription of genes such as tyrosine aminotransferase (TAT) and phosphoenolpyruvate carboxykinase (PEPCK) (Jantzen et al., 1987; Hanson and Reshef, 1997) while a lot of the anti-inflammatory properties are often attributed to negative transcriptional effects on the expression of inflammatory genes (Reichardt and Schutz, 1998). Anti-inflammatory and immunosuppressive actions are the primary pharmacological benefits of glucocorticoids. However, they also find widespread use in chemotherapeutic regimes in cases of leukemias, lymphomas and other types of cancers (Yudt and Cidlowski, 2002).

The Munck laboratory in 1968 identified GR in rat thymic lymphocyte cytosol (Munck and Brinck-Johnsen, 1968; Pratt and Toft, 1997). Molybdate and other transition metal oxyanions have been shown to stabilize the interaction of HSP90 with the receptor (Nielsen et al., 1977). Physiological response and sensitivity to glucocorticoids varies among different species, individuals, tissues, cell types and even stages of the cell cycle (Hsu and DeFranco, 1995). The only known gene for GR in the case of humans is located on chromosome 5 q11-q13, (Hollenberg et al., 1985) and comprises nine exons, with the coding region beginning from exon 2. The promoter region lacks TATA and CCAAT but does have multiple GC boxes and several unique binding sites for the transcription factors Sp1, AP-1, NF-KB and GR itself (Breslin et al., 2001). At least three unique promoters have been identified for both human as well as mouse GR (Nobukuni et al., 1995) giving rise to numerous mRNAs with unique 5’ untranslated regions.
Alternate splicing of the hGR gene has been reported to give rise to two major products. Human GR\(\alpha\) is a single polypeptide chain of 777 amino acids localized in the cytoplasm in the untransformed state and identified as the primary mediator of glucocorticoid action (Hollenberg et al., 1985). The other isoform hGR\(\beta\), is generated by alternative splicing of the ninth and final exon resulting in a protein that only varies in the C-terminal sequence. This isoform is transcriptionally inactive being unable to bind agonists or antagonists in all systems tested to date (Oakley et al., 1996). Despite its association with HSP90, hGR\(\beta\) is localized in the nucleus and reports have confirmed its dominant negative effect on hGR\(\alpha\)-mediated transactivation (Oakley et al., 1999).

Several isoforms of GR are also produced by alternative translation initiation. GR-A represents the protein product with a molecular weight of about 94 kDa, generated by translation from the first AUG codon. This codon is located within a weak kozak translation initiation consensus sequence resulting in leaky ribosomal scanning and translation initiation from downstream AUG codon(s) (Yudt and Cidlowski, 2001). The 91kDa form of GR referred to as GR-B is generated as a consequence of utilization of one of downstream codons. The two forms differ in their trans-activation potential with GR-B being nearly twice as efficient as GR-A (Yudt and Cidlowski, 2001).

In the classical paradigm, hormone-free GR is primarily cytoplasmic in its distribution. Intracellular studies performed using GR fused to GFP showed the unoccupied receptor to be completely cytoplasmic with rapid translocation to the nucleus upon treatment with dexamethasone or RU486 (Htun et al., 1996). Indirect immunofluorescence experiments on cultured cells revealed a punctate, nuclear
distribution of ligand-bound GR (Steensei et al., 1995). Under unliganded conditions, GR complexed with HSP90 shows a dynamic shuttling between cytoplasmic and nuclear compartments (Hache et al., 1999). This gave birth to the speculation that predominant cytoplasmic localization of naïve GR complexes would either require active cytoplasmic retention as reported for other proteins (Li et al., 1994), or changes in the relative rate of activities of nuclear localization and nuclear export signals (Defranco et al., 1995). The role of NLS in distribution of unoccupied steroid receptors between cytoplasmic and nuclear compartments still remains a much debated issue. In this work, we provide evidence to show that steroid receptor localization under hormone-free conditions may be
influenced by the nature of the TPR protein present in the complex. Even in the presence of ligand, GR is not constantly bound to its hormone response element (HRE) on DNA but is involved in a rapid exchange with regulatory elements (McNally et al., 2000). More recently, rapid exchange of GR at a tandem array of mouse mammary tumor virus (MMTV) promoter sites was shown to involve chaperones as well as proteasomes and longer GR residence correlated with increased transcription (Stavreva et al., 2004).

Pratt and co-workers determined the composition of the mature GR heterocomplex and showed that it contained two molecules of HSP90 forming the backbone of the complex along one molecule of p23 (Pratt and Toft, 1997). Interaction of GR with HSP90 involved a highly conserved region of the steroid receptor called the Signal Transduction Domain (STD). The HSP90 binding co-chaperones, some of which contain TPR domains, compete for the single binding site generated by the HSP90 dimer. The TPR is characterized by a 34-amino acid consensus sequence found in tandem repeats of varying number (Blatch and Lassle, 1999). Four major TPR proteins found to enter the steroid receptor complex include FKBP51, FKBP52, Cyp40 and PP5 (Figure. 2) (Peattie et al., 1992; Ratajczak et al., 1993; Smith et al., 1993a; Pratt and Toft, 1997).

Based on some recent findings from our laboratory, we now have a new model highlighting the early stages of “hormone-induced” GR activation. It deviates from the classical model in the initial stages of signaling, prior to the translocation of the hormone-bound receptor into the nucleus. This model has identified the swapping of immunophilin within the receptor heterocomplex as the first
consequence of ligand binding to the cytoplasmic receptor (Figure. 3). Addition of hormone caused GR complexes to shift from those that were predominantly bound to FKBP51 to complexes where FKBP52 was the principal TPR.

“Hormone-induced” switching of immunophils is followed by concomitant recruitment of the motor protein, dynein (Davies et al., 2002). Since this event correlated with translocation of the complex to the nucleus leading to its final dissociation and conversion to, high affinity chromatin binding form, TPR exchange can be viewed as the first step in hormonal activation of steroid receptors.
STRUCTURE AND FUNCTION OF PROGESTERONE RECEPTOR

The human progesterone receptor exists in two isoforms. PR-A (94kDa) is the truncated version of PR-B (120kDa), lacking the first 164 amino acids at the N-terminus (Figure 4) (Kastner et al., 1990). The two isoforms have similar hormone and DNA binding abilities but differ in their transcriptional activities. PR-B has an additional activation function domain (AF-3) embedded within the first 164 residues at the N-terminal, which might account for its stronger activation potential (Richer et al., 2002). An inhibitory domain having trans-repressive functions has been mapped to the first 140 N-terminal amino acids of PR-A. Although this sequence is present in both isoforms, it is active only in PR-A suggesting a suppressive role played by the specific amino acids in the N-terminal sequence of PR-B. Studies using GFP fusions of PR-A and PR-B to determine their intracellular localization revealed that although PR-B is primarily nuclear in the unliganded state, it does have a small cytoplasmic component and the addition of ligand causes this component to rapidly translocate to the nucleus (Lim et al., 1999). However, PR-A is predominantly nuclear even in the absence of ligand. The 164 amino acids
acid N-terminal extension of PR-B may act as a cytoplasmic retention sequence, thereby accounting for the observed difference in localization of the two isoforms.

Both in the presence and absence of ligand, GR and PR exhibit a dynamic behavior by continuously shuttling between nucleus and cytoplasm (Tyagi et al., 1998; Hache et al., 1999). Thus, the equilibrium distribution of these receptors at any given point of time represents the net difference in the rates of nuclear import and export steps. Sequences responsible for nuclear localization of GR and PR following addition of hormone have been mapped to two clusters of basic amino acids in the DBD and another in the hinge region (Ylikomi et al., 1992). The LBD has been implicated in the differential distribution of the two receptors in the absence of hormone (Ylikomi et al., 1992). Detailed analysis using LBD mutants of wild type and chimeric forms of GR and PR tagged with an enhanced GFP moiety revealed that 87 amino acids (helices 1-5) localized in the N-terminal of the LBD determined subcellular localization. Mutations in helices 1 through 5 had no effect on ligand-binding since specificity for this function were conferred by helix 7, larger $\beta$-turn helix 6-helix 7 domain and helix 3 (Wan et al., 2001). Besides determining localization, this region plays a role in binding chaperone proteins as well as interacting with nuclear trafficking proteins thereby affecting the equilibrium distribution of the receptors.

The functional consequences of differential localization have not been clearly evaluated in the case of either receptor. Several reports indicate that localization of PR may be regulated in a developmental or tissue-specific fashion (Leslie et al., 2005). In most endometrial adenocarcinomas of all grades (well, moderately and poorly
differentiated) PR-A was nuclear and PR-B was distributed between the nuclear and cytoplasmic compartments with exclusion from the nucleus observed only in the case of poorly differentiated tissue samples. Nuclear localization of PR-B was prominent in highly differentiated tumors. The biological implications of the above-mentioned distribution are well corroborated with other findings such as the presence of a strong Src-homology-3 domain (SH3) in both PR isoforms, which allow receptor interaction in vitro with several cell signaling molecules, such as Src kinases (Leonhardt et al., 2003). Both PR-A an PR-B demonstrate this interaction, but it is speculated that predominant nuclear localization of unliganded PR-A might allow the receptor to assert its ligand-independent nuclear effects and restrict its role in cytoplasmic signaling. PR-B, on the other hand, might be actively involved in nongenomic cytoplasmic actions in endometrial cancers (Leslie et al., 2005). These observations heighten the importance of a thorough understanding of steroid receptor distribution and trafficking.

**HSP90 STRUCTURE AND FUNCTION**

HSP90, which is recognized as the central protein of the steroid receptor assembly and maturation pathway, accounts for nearly 1-2% of cytosolic proteins making it one of the most abundant and highly conserved essential eukaryotic proteins. It performs a host of housekeeping functions under unstressed conditions besides its role in stressed cells. HSP90 is a phosphorylated dimer (Lees-Miller and Anderson, 1989) containing two to three phosphate molecules per monomer with a strong tendency to oligomerize in the presence of divalent cations, nucleotides, and at higher HSP90 concentrations (Minami et
al., 1993). The two isoforms of HSP90; HSP90-α and HSP90-β share 76% structural similarity (Moore et al., 1989). HSP90-β is the larger of the two isoforms and somewhat less inducible than its smaller counterpart. Being one of the “stickiest” proteins, it interacts with a host of other proteins, which include protein kinases like casein kinase II (CK-2) (Dougherty et al., 1987), phosphatases such as PP5 (Silverstein et al., 1997), nuclear hormone receptors (Pratt and Toft, 1997), tubulin (Sanchez et al., 1988), calmodulin (CaM) (Minami et al., 1993) and even the proteasomal machinery (Tsubuki et al., 1994).

The tertiary structures of the N-terminal domains of human (Stebbins et al., 1997) and yeast (Prodromou et al., 1997) HSP90 are almost identical consisting of eight-stranded β-sheet covered on one side by α-helices. From the center of the helical sheet, a deep pocket penetrates to the surface of the buried β-sheet forming a binding site for ATP/ADP as well as for the antitumor HSP90-specific drug, geldanamycin (GA) (Stebbins et al., 1997). This site possibly overlaps with the binding site for another HSP90-binding antibiotic, radicolol (Soga et al., 1998). The unique structural feature of the nucleotide-binding site in the N-terminal region allows HSP90 to be classified as a member of the GHKL superfamily of ATPases (Dutta and Inouye, 2000). Other members of this family include bacterial DNA gyrase, DNA mismatch- repair protein MutL and several bacterial histidine kinases, all of which require ATP binding and phosphotransferase activity. The presence of an ATP-binding site allows HSP90 to undergo autophosphorylation, which in turn elicits conformational changes in the structure of client proteins required for their activation (Csermely and Kahn, 1991;
Csermely et al., 1993; Grenert et al., 1999). The highly charged hinge (central) region of HSP90 permits interaction with steroid receptors, protein kinase CK-II (Tbarka et al., 1993; Miyata and Yahara, 1995) and houses the NLS.

The C-terminal domain harbors the dimerization site along with a binding site for calmodulin (Minami et al., 1993; 1994). Co-chaperones of HSP90, including TPR proteins and Hop, compete for a single binding site generated at the C-terminal end of the dimer (Chen et al., 1998; Carrello et al., 1999). The highly conserved MEEVD sequence and an additional hydrophobic region within the dimerization domain act as critical elements for TPR binding (Chen et al., 1998).

Receptor function modulation by HSP90 co-chaperones was demonstrated by expressing human FKBP51 and FKBP52 in yeast (Riggs et al., 2003). Recruitment of FKBP52 by the receptor complex potentiated steroid binding as well as reporter gene activity. Further experiments confirmed the fact that FKBP52-mediated increase in GR hormone-binding required an obligate interaction with HSP90 along with the PPIase activity of FKBP52.

The pivotal role of HSP90 in rapid ligand-dependent translocation of GR is supported by numerous observations. Evidence for this emerged when, in the case of both endogenous and transfected GR (GFP GR), treatment of cells with GA slowed down the rate of receptor translocation to the nucleus from $t_{1/2} \sim 4.5$ min to $t_{1/2} \sim 45$ min (Czar et al., 1997). The loss of rapid HSP90-dependent movement in the presence of cytoskeletal inhibitors such as colcemid, established an unequivocal requirement for the intact cytoskeletal framework. (Galigniana et al., 1998). Treatment of intact cells with GA led
to rapid loss of hormone-binding and accelerated degradation of GR (Whitesell and Cook, 1996). Axons and dendrites, being specialized cytoplasmic extensions, do not support protein movement by diffusion alone. Therefore, in the presence of GA, ligand-induced retrograde movement is inhibited confirming the dependence on HSP90 for this process (Galigniana et al., 2004c). Similar observations reported in the case of AR confirmed the role of HSP90 in ligand-dependent movement of nuclear receptor family members that form persistent complexes with this protein (Thomas et al., 2004).

However, the role of HSP90 has not just been confined to hormone-binding competence and translocation of naïve cytoplasmic receptors, but recent reports also confirmed its role in maturation of recycled receptors. In digitonin-permeabilized cells, hormone-binding of recycled nuclear GR was inhibited following the treatment of these cells with GA (Liu and DeFranco, 1999). A potential role of this chaperone also has been uncovered in sub-nuclear trafficking of nuclear receptors. Hormone withdrawal in the presence of GA leads to release of bound steroid by the receptors, but their subsequent release from chromatin is drastically inhibited implicating the involvement of HSP90 in this process (Tanenbaum et al., 1998). Some recent findings suggest a possible role of HSP90 in quality control. The exact region involved in the binding of HSP90 to the proteasome (Tsubuki et al., 1994) is yet to be confirmed, but a highly charged KEKE motif in the linker region may be involved (Realini et al., 1994).

The study of steroid receptor assembly using reticulocyte lysate led to the discovery of p23, another essential co-chaperone of steroid receptor complexes. It is a highly acidic 23-kDa protein capable of binding to only the ATP-dependent conformation
of HSP90 (Sullivan et al., 1997). Although a unique p23 interaction domain is yet to be identified, it has been demonstrated that residues outside of nucleotide/GA-binding site are required (Grenert et al., 1997). The main function of p23 identified in steroid receptor complexes limits its role to a type by passive chaperoning of helping in the stabilization of the mature steroid receptor heterocomplex (Dittmar et al., 1997).

ROLE OF TPR PROTEINS IN STEROID RECEPTOR COMPLEX

The HSP90 co-chaperones found in steroid receptor complexes include the larger members of FK506 and CsA families of immunophilins. Besides possessing PPIase activity these proteins also compete for the single binding site at the C-terminal of HSP90 and the interaction of these TPR proteins with receptor complexes is strengthened by molybdate (Owens-Grillo et al., 1995). The commonly observed receptor-associated immunophilins include FKBP52; FKBP51 and Cyp40 (Figure 5). PP5 (Chinkers, 1994),

![FIGURE 5. DOMAIN ORGANIZATION OF TPR PROTEINS](image-url)
which binds to FK506 with low affinity and shows sequence homology with FKBP PPIase domain (Silverstein et al., 1997) competes with the other immunophilins for the binding-site on HSP90. Thus, four major steroid receptor complexes can be distinguished based on the TPR content. The TPR is characterized by a sequence of 34-amino acids found in tandem repeats of varying number (Blatch and Lassle, 1999). The key structural attribute of TPR motifs relies on its ability to form antiparallel alpha helical hairpins that generate a grooved surface enabling other polypeptides to bind. Although the TPR region stands out as one of the important structural features of these proteins, they also have other functional domains such as the PPIase domain or the CaM binding site on FKBP52. The rapid exchange of TPR proteins generates receptor complexes that may be bound to any of the available TPR proteins, but previous work as well as our current findings suggests that these proteins do not enter complexes strictly based on their relative abundance. Steroid receptor complexes show a distinct preference pattern for TPR proteins (Barent et al., 1998) with hormone-binding leading to a rapid shift in selectivity (Smith et al., 1993a; Davies et al., 2002). The following section describes each member of the GR heterocomplex and cites evidence in favor of their roles in receptor function regulation.

**STRUCTURE AND FUNCTION OF FKBP51**

FKBP51, also termed p54, FKBP54 or FKBP5 was first observed in PR complexes purified by immunoaffinity chromatography (Smith et al., 1990). This protein shares approximately 75% sequence similarity with FKBP52 (Nair et al., 1997). The
NMR structure for full length FKBP51 (Sinars et al., 2003) revealed TPR domains that interact with the highly conserved MEEVD sequence in the C-terminal region of HSP90. The functional domains which lie upstream of the TPR domains include two N-terminal domains. The first domain of the pair (FK1) has PPIase activity and binds the immunosuppressant drug FK506; the second domain (FK2) lacks PPIase or drug-binding activity, despite its structural similarity to FK1 (Figure. 5) (Nair et al., 1997; Sinars et al., 2003). Subcellular localization of FKBP51 is not a well researched area. However, using a rabbit polyclonal antibody, we show FKBP51 to be uniformly distributed between cytoplasmic and nuclear compartments in L929 and WCL2 cells, while in COS-1 cells a predominant nuclear localization was detected (Fig. 2 manuscript).

FKBP51 was shown to bind both GR and PR complexes, although its association with PR was highly sensitive to hormone with molybdate being unable to prevent dissociation of FKBP51 from liganded PR complexes (Smith et al., 1990). In spite of 60% identity and 75% similarity in amino acid sequence along with conservation of most of the domain residues, mutations in the C-terminal half of HSP90 produced differential effects on binding of FKBP51 versus FKBP52 (Chen et al., 1998). A comparison of co-immunoprecipitation with HSP90, using a set of truncation mutants of FKBP51 and FKBP52, identified regions outside the core TPR sequence (260-400) that influenced their binding to HSP90. In the case of FKBP51, the final 30 residues in the C-terminal were crucial in enhancing its binding to HSP90 (Cheung-Flynn et al., 2003). In experiments focusing on the association of PR with FKBP51, site-directed mutagenesis
of unique residues in the FK2 domain abrogated association with PR without affecting its HSP90 binding ability (Sinars et al., 2003).

An increasing body of evidence indicates expression of several TPR proteins is hormonally regulated. Glucocorticoid resistance in New World primates has been attributed to their constitutively low expression of FKBP52 (Reynolds et al., 1999; Scammell et al., 2001). Expression of FKBP51 is up-regulated by glucocorticoids (Baughman et al., 1995; 1997), progestins (Kester et al., 1997; Hubler et al., 2003) and current reports have documented alteration in FKBP51 levels under the influence of androgens (Amler et al., 2000; Febbo et al., 2005). Hormone-induced changes in equilibrium levels of TPR proteins may alter receptor complex composition with potential implications on receptor localization and function. FKBP5, which encodes FKBP51, contains 10 exons and 9 introns spanning approximately 9kb of genomic DNA with an additional intron located in the 5’-noncoding sequence (Scammell et al., 2003). Glucocorticoid and progestin regulated expression of FKBP5 has now been identified to occur at least in part by distal intronic HREs (Hubler and Scammell, 2004).

Understanding the contribution of HSP90 co-chaperones in the development of glucocorticoid and progesterone resistance has been one of the major hallmarks in steroid receptor biology. Novel insights into the molecular basis of cortisol resistance have emerged from studies on neotropical primates such as squirrel monkeys. The reduced hormone-binding affinity of GR from squirrel monkey lymphocytes (SML) was attributed to 13-fold higher expression levels of FKBP51 compared to human lymphocytes (Reynolds et al., 1999). Development of glucocorticoid resistance and the
reduced hormone-binding affinity displayed by GR in this species has been linked to the recruitment of co-chaperone FKBP51 into the receptor complexes (Denny et al., 2000). However, in the presence of FK506 and rapamycin, a dramatic increase in the receptor’s hormone-binding in cytosols from SML correlated with the loss of FKBP51 from receptor complexes and its inability to reassemble with receptor in the presence of drugs (Smith et al., 1993b; Denny et al., 2000).

The pronounced inhibitory effect of squirrel monkey FKBP51 on GR hormone-binding compared to human FKBP51 are likely to be mediated by differences in the amino acid residues found in the N and C terminal regions of this protein (Scammell et al., 2001). Besides differences in the sequence of FKBP51, transcriptionally incompetent GR has a definite contribution towards the development of glucocorticoid resistance in these animals (Westberry et al., 2006). Currently, no direct interacting partner of FKBP51 other than HSP90 has been reported. Therefore, the obligate requirement for HSP90 in mediating its inhibitory effect was of little surprise.

Apart from a decrease in receptor’s hormone-binding affinity, over-expression of FKBP51 in HeLa cells disrupted translocation of liganded (Wochnik et al., 2005) GR. It is speculated that FKBP51 bound to GR heterocomplexes either failed to interact with the transport machinery or the interaction was unproductive. Thus, FKBP51 may function as a part of a feedback loop to attenuate hormone response in conditioned cells and tissues. Possible mechanisms may revolve around the ability of this protein to alter hormone binding or affect transcriptional activity by preventing the translocation of unliganded cytoplasmic receptors. Our findings implicate a role for FKBP51 in determining
localization subcellular of GR and PR. We do not rule out the possibility of an overlapping role with PP5, at least in the case of localization. However, to completely delineate the role FKBP51 in GR signaling and unravel some of the intricacies would involve using FKBP51 knock out (KO) animals. Physiological significance of receptor associated immunophilins and possible redundancies in TPR functions would be best addressed using these animal models.

FKBP52 STRUCTURE AND FUNCTION

FKBP52, also termed as p59, HSP56, p50, HB1, FKBP59 or FKBP4 was first identified in glucocorticoid receptor complexes (Figure. 5) (Tai et al., 1986). In IM-9 cells, a 56-kDa protein interacting with GR and inducible by both heat and chemical stress was later identified to be the same protein as FKBP52, although it was relabeled as HSP56 (Sanchez et al., 1990b). Crystallographic analyses and sequence studies have revealed four major domains in FKBP52. The N-terminal domain has PPIase activity. Immunosuppressant drugs like FK506, rapamycin etc. have been shown to bind to this region. Unlike the lower molecular weight immunophilin FKBP12, FKBP52 does not inhibit calcineurin when bound to these drugs (Lebeau et al., 1994; Davies and Sanchez, 2005). Following the N-terminal region is a domain, which has PPIase-like activity but does not bind FK506. This region has a consensus ATP/GTP-binding sequence between amino acids 199 and 222 (Callebaut et al., 1992) of unknown function. Protein kinase CK-2 driven phosphorylation of Thr-143 in the hinge region prevents FKBP52 from binding to HSP90 (Miyata et al., 1997). The hinge region also has an eight amino acid
sequence that is electrostatically complementary to the NLS on the receptor. Antibodies against this sequence reduce the rate of translocation of liganded GR to the nucleus (Czar et al., 1995). The three TPR domains allow FKBP52 to interact with HSP90. Following the TPR domains are two putative CaM containing PEST sequences, which are positioned towards the C-terminal end of the molecule (Massol et al., 1992). The gene (FKBP4) encoding human FKBP52 protein has been mapped to the short arm of chromosome 12 (Bermingham et al., 1998). It contains 10 exons and 9 introns with the introns significantly shorter than those of FKBP5 (Scammell et al., 2003).

The role of FKBP52 in GR transactivity has been well documented. So far, no known homologues of FKBP51 and FKBP52 have been reported in yeast making it an ideal setting for dissecting the role of TPR proteins in modulation of steroid receptor activity. Expression of human FKBP52 in yeast potentiated GR-mediated reporter gene activity (Riggs et al., 2003), while in the mammalian system, elevated levels of FKBP52 had no effect on receptor function except for mitigating the inhibitory effect of FKBP51 (Wochnik et al., 2005). Although HSP90 acts as the adaptor molecule in the interaction between GR and FKBP52, it is speculated that these immunophilins, by virtue of their intrinsic PPIase activity, may be able to modify the GR LBD into distinct conformations with higher or lower affinity for the hormone (Riggs et al., 2003).

The significance of FKBP52’s role in nuclear translocation became clear when receptor trafficking to the nucleus was impeded by antibodies against the highly conserved negatively charged 8 amino acid sequence in the hinge region of this protein (Czar et al., 1995). FK506 failed to affect receptor trafficking to the nucleus, suggesting
that the PPIase activity is not required in this process (Czar et al., 1995). Its role in trafficking is easily substantiated by a battery of literature suggesting its association with motor protein dynein as well as its co-localization along the microtubules (Czar et al., 1994; Davies et al., 2002). Interaction of FKBP52 with cytoplasmic dynein occurs via its PPIase domain and in a very elegantly designed experiment using rabbit reticulocyte dynein was shown to co-immunoprecipitate with FKBP52. This interaction was only competed by a fragment of FKBP52 containing PPIase domain, and not by the TPR domain fragments (Silverstein et al., 1999). The above mentioned observations support the notion that FKBP52 targets retrograde movement of GR along the microtubules by facilitating an indirect interaction between the receptor and dynein.

The immunofluorescence pattern produced by the UPJ56 antibody against FKBP52 showed that substantial amounts of FKBP52 co-localized with tubulin in the cytoplasm of intact WCL2 and rat pulmonary endothelial cells (Czar et al., 1994). In LLC-PK1 porcine kidney cells, a fibrillar staining pattern was indicative of an association with the cytoskeleton (Ruff et al., 1992). In L929, WCL2 and COS-1 cells, we also observed an immunofluorescence pattern that was predominantly cytoplasmic and localized along the cytoskeletal network (Czar et al., 1994; Ruff et al., 1992; Fig. 2 manuscript). The cytoskeletal staining of FKBP52 supports its role in receptor trafficking to the nucleus. In MCF-7 cells, although FKBP52 was localized throughout the cytoplasm, a more abundant distribution was detected in the nucleus (Mark et al., 2001). The differences observed in the distribution of FKBP52 amongst different cell types could entail distinct distribution patterns inherent to a particular cell type. Another
possibility is that different antibodies against FKBP52 bind to unique epitopes that are masked in one locus but open in another.

The biological roles of FKBP52 extend beyond its well documented role in receptor transactivation and translocation. Glucocorticoid resistance in New World primates has been attributed to their constitutively low expression of FKBP52 (Reynolds et al., 1999; Scammell et al., 2001). In immune cells, FKBP52 acts as a transcriptional regulator and is actively involved in repressing the activity of interferon regulatory factor-4 (IRF-4) (Mamane et al., 2000). This protein also has been implicated in the neuroprotective effects of FK506 and in the cardiostrophic effects of cardiotrophin-1 (Gold et al., 1999; Railson et al., 2001). A regulatory role of this protein was recently noted in adeno-associated virus type-2 mediated transgene expression (Qing et al., 1998; Qing et al., 2001).

Understanding the diverse role of FKBP52 in physiological processes is an ongoing effort in many laboratories. Current evidence highlights the importance of this co-chaperone in regulating tissue-specific hormone action of PR-A and the indispensable role of FKBP52 in conditioning the uterus for embryo implantation (Tranguch et al., 2005; Yang et al., 2006). Further investigations using a proteomic approach identified FKBP52 as one of the downstream effector molecule in the signaling pathway controlled by Hoxa 10, a transcription factor regulating decidualization and embryo implantation in the uterus (Daikoku et al., 2005). Studies in FKBP52 KO male mice revealed that this protein plays a critical role in male fertility by controlling AR, mediated signaling and
physiology, with its loss resulting in hypospadias as well as prostate dysgenesis (Cheung-Flynn et al., 2005; Yong et al., 2007).

Our findings suggest a strong involvement of FKBP52 in determining cellular localization of PR-B since the loss of this protein led to cytoplasmic localization of an otherwise primarily nuclear receptor. In our cell systems PR-B also recruited FKBP51, which we speculate to be the TPR of choice in the absence of FKBP52. We have not investigated how localization of PR-A might be affected in the absence of FKBP52. Since current reports support an association between PR-A and FKBP52, we speculate that the receptor would behave much like PR-B in the absence of this protein and adopt a cytoplasmic localization. It would be interesting to identify the TPR protein being recruited into PR-A heterocomplexes in these KO cells. This would reveal alternate preferences of the receptor, if any, or reiterate the role of equilibrium concentrations of TPR proteins in determining receptor localization.

**Cyp40 STRUCTURE AND FUNCTION**

Cyp40 was first identified as a part of estrogen receptor (ER) complexes (Ratajczak et al., 1993) and later isolated from the soluble fraction of calf brain along with Cyp18 by adsorption on a CsA affinity column followed by elution with CsA (Kieffer et al., 1992). The structure of Cyp40 is similar to that of FKBP51 and FKBP52 except that it contains a single active PPIase domain, three TPR domains that allow interaction with HSP90 and a CaM site at the C-terminus (Figure. 5) (Ratajczak et al., 1993; Hoffmann and Handschumacher, 1995). Apart from ER complexes, Cyp40 has
been reported to be a constituent of native GR complexes in small amounts as well as a part of cell-free reconstituted GR and PR heterocomplexes (Owens-Grillo et al., 1995; Owens-Grillo et al., 1996). In the cell systems we used in this study, namely, L929, WCL2 and COS-1, Cyp40 was undetectable in both GR and PR heterocomplexes (Fig. 4A and 7A manuscript).

Hormonal regulation of TPR protein expression also has been reported in the case of Cyp40. In MCF-7 breast cancer cells, up-regulation of Cyp40 expression by the mitogenic influence of estradiol closely mimicked that of FKBP52 (Kumar et al., 2001). Based on existing reports, subcellular distribution of Cyp40 appears to be very cell-type specific. A predominantly nucleolar localization was reported in rat pulmonary endothelial cells (Owens-Grillo et al., 1996) with similar findings in the MCF-7 cells, where a distinct punctate pattern in the nucleus suggested similar nucleolar localization (Mark et al., 2001). The change in the cellular distribution pattern of Cyp40 in MCF-7 cells from a predominantly nuclear distribution to a strict nuclear localization in response to heat shock led to its identification as a heat shock protein (Mark et al., 2001). To the contrary, an almost exclusively cytoplasmic localization was observed in LLC-PK1 cells (Yokoi et al., 1996). The immunofluorescence pattern produced by a rabbit polyclonal antibody (PA3-022) against Cyp40 revealed a predominantly cytoplasmic distribution in L929, WCL2 and COS-1 cells (Fig. 2 manuscript).

Even though no known homologues of FKBP51 and FKBP52 have been reported in yeast, Cyp40 homologues have been identified in a yeast strain (S. cerevisiae) (Duina et al., 1996). Cpr6 and Cpr7 are the two homologues of which Cpr6 is more closely related
to Cyp40 (Duina et al., 1996). Their binding affinity for HSP90 is comparable, although Cpr6 has higher catalytic activity while Cpr7 is a better chaperone (Mayr et al., 2000). Moreover, the chaperoning activity of Cyp40, like FKBP52, is independent of the PPIase activity, and, consequently, is not subject to CsA suppression (Freeman et al., 1996). The interaction of Cyp40 with cytoplasmic dynein at the PPIase domain reflects an apparent redundancy in the role of TPR proteins in nuclear transport of steroid receptors (Galigniana et al., 2002).

**PP5 STRUCTURE AND FUNCTION**

The reversible phosphorylation of proteins regulating several vital cellular processes such as cell cycle progression, DNA replication, transcription and protein translation are catalyzed by kinases and phosphatases (Kurosawa, 1994). PP5 is one such serine/threonine phosphatase present in most eukaryotic cells and tissues. This protein was identified in a yeast two-hybrid screen based on the interaction of its TPR domain with atrial natriuretic peptide (ANP) (Chinkers, 1994). The C-terminal end of PP5 contains the phosphatase domain comprised of regulatory and catalytic subunits (Figure 5). Reports state that the catalytic subunit of PP5 has a 42-43% similarity with those of PP1, PP2A and PP2B (Barton et al., 1994). The region linking the N-terminal TPR domains to the C-terminal phosphatase domain has about 55 residues and shows a 50% amino acid homology with the central PPIase domain of FKBP52 (Silverstein et al., 1997). The distinctive biochemical and biological properties of PP5 are attributed to the four TPR domains that occupy the N-terminal region rather than the C-terminal seen in
other immunophilins and fulfill most of the regulatory and targeting functions (Becker et al., 1994; Blatch and Lassle, 1999).

PP5 is recruited into receptor heterocomplexes via its TPR domain interaction with HSP90 (Silverstein et al., 1997). Over-expression of the TPR domain of a dominant negative mutant of PP5 in CV-1 cells inhibited glucocorticoid-induced gene activity (Chen et al., 1996). In a different study, suppression of PP5 expression enhanced GR activation along with hyperphosphorylation of p53 and a subsequent increase in the expression of p21-cyclin-dependent kinase inhibitor protein (Zuo et al., 1999). Similar observations in MCF-7 cells suggest a role of PP5 in promoting estrogen-induced cell growth while inhibiting GR-induced cell signaling cascades, leading to growth arrest (Urban et al., 2001).

The ability of PP5 to interact with dynein (Galigniana et al., 2002) quite easily justifies its role in nucleocytoplasmic shuttling of GR (Dean et al., 2001), as inhibition of the phosphatase activity by okadaic acid and oligonucleotide suppression altered subcellular localization of the receptor (DeFranco et al., 1991). In yeast over-expression of PP5 alone had no bearing on GR hormone-binding and reporter gene activity. Co-expression of PP5 with FKBP52 failed to abrogate the potentiation in hormone-binding mediated by the latter, suggesting a lack of competition for the binding site on HSP90 (Riggs et al., 2003). However, in the presence of FK506 in mammalian systems, the increase in the receptor's hormone-binding capacity and decrease in total number of hormone-binding sites (βmax) correlated well with an exchange of FKBP51 for PP5 within GR heterocomplexes (Davies et al., 2005).
The localization of PP5 shows that this enzyme is present in both subcellular compartments (Chen et al., 1994; Ollendorff and Donoghue, 1997; Borthwick et al., 2001). In the cell lines we tested, localization of PP5 was primarily cytoplasmic supporting its predicted role in nuclear transport of cytoplasmic receptors (Fig. 2D manuscript). The autoinhibitory role of the TPR domain is responsible for the low basal phosphatase activity of this enzyme and a 50-fold increase was observed with the removal of this domain (Chen and Cohen, 1997). The unusually low phosphatase activity of PP5 led to a search for other potential physiological activators. Polyunsaturated fatty acids such as arachidonic acid activated PP5 in vitro, but at concentrations that were not physiologically relevant (Chen and Cohen, 1997; Skinner et al., 1997). Currently, the identity of physiological activators of PP5 is still a mystery. However, future possibilities include screening of other polyunsaturated fatty acids that might help to identify a novel link between fatty-acid metabolism and the enzymatic role of PP5.

The diverse biological roles of PP5 range from modulation of GR signaling (Chen et al., 1996), promotion of cell growth (Zuo et al., 1998) to cell cycle regulation (Weinian Shou unpublished data). PP5 performs a multitude of functions related to cell cycle regulation, which include cellular proliferation, migration, differentiation, survival and check point regulation (Ollendorff and Donoghue, 1997). Several lines of evidence have implicated an active involvement of PP5 in ataxia telangiectasia mutated (ATM) and ATR (ATM-related) pathways controlling cell cycle checkpoints (Wechsler et al., 2004; Zhang et al., 2005). Recently, the physiological impact of loss of PP5 in cell cycle regulation was demonstrated using MEF cells generated from PP5-deficient animals.
Although embryonic development and postnatal life are not affected in these animals, PP5 KO MEF cells displayed a significant defect in the G2/M DNA damage checkpoint when exposed to ionizing radiation (IR). The activity of cell cycle checkpoint master controller, ATM, was reduced in these cells leading to a subsequent reduction in the phosphorylation levels of ATM substrates (Weinan Shou *unpublished data*). Identification of direct targets of PP5 in ATM survival mechanisms may further our understanding of how loss of this protein might accelerate genomic instability leading to cancer development.
Specificity of Tetratricopeptide Repeat Protein Interaction and Subcellular Localization is Determined by the Ligand-binding Domains of Glucocorticoid and Progesterone Receptors

Ananya Banerjee¹, Irene M. Wolf¹, Weidong Yong², Weinian Shou² and Edwin R. Sanchez¹*

¹Department of Physiology, Pharmacology, Metabolism and Cardiovascular Sciences
University of Toledo College of Medicine, 3035 Arlington Avenue, Toledo, OH 43614, USA.
²Herman B Wells Center for Pediatric Research, Section of Pediatric Cardiology,
Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN 46202, USA.

Short title: TPRs and Steroid Receptor Localization

*Address all correspondence to: Edwin R. Sanchez, Department of Physiology & Pharmacology, Metabolism and Cardiovascular Sciences, University of Toledo College of Medicine, 3035 Arlington Avenue, Toledo, OH 43614-5804, edwin.sanchez@utoledo.edu, (419) 383-4182, FAX (419) 383-2871

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SUMMARY

The TPR proteins FKBP52, FKBP51, Cyp40 and PP5 are found in steroid receptor (SR) complexes but their receptor-specific preferences and roles remain unresolved. We have undertaken a systematic approach to this problem by examining the contribution of all four TPRs to the localization properties of glucocorticoid (GR) and progesterone (PR) receptors. The GR of L929 cells were found in the cytoplasm in a complex containing PP5 and FKBP51, while GR of WCL2 cells was nuclear and contained PP5 and FKBP52. Cyp40 did not interact with GR in either cell line. To test whether FKBP interaction determined localization, we over-expressed Flag-tagged FKBP51 in WCL2 cells and Flag-FKBP52 in L929 cells. In WCL2 cells, GR showed a shift to greater cytoplasmic localization that correlated with recruitment of Flag-FKBP51. In contrast, Flag-FKBP52 was not recruited to GR of L929 cells and no change in localization was observed, suggesting that both cell-type specific mechanisms and TPR abundance contribute to the SR/TPR interaction. As a further test, GR-GFP and PR-GFP constructs were expressed in COS cells. GR-GFP localized to the cytoplasm, while PR-GFP was predominantly nuclear. Similar to L929 cells, GR in COS interacted with PP5 and FKBP51, while PR interacted with FKBP52. Analysis of GR/PR chimeric constructs revealed that the ligand-binding domain of each receptor determines TPR specificity and localization. Lastly, we analyzed GR and PR localization in cells completely lacking TPR. PR in FKBP52 KO cells showed a shift to the cytoplasm, while GR in FKBP51 KO cells shifted to the nucleus. GR and PR localization were unaffected in PP5 KO cells. Our results demonstrate that SRs have distinct preferences for TPR proteins – a property
that resides in the LBD and which can now explain long-standing differences in receptor
subcellular localization.

**INTRODUCTION**

Steroidal control of reproduction, metabolism and other physiological processes
requires the activation of steroid receptors (SR), which serve as regulators of differential
gene expression (Mangelsdorf et al., 1995; Beato and Klug, 2000). Although many of the
diverse effects of SRs can be explained based on the intricacies of transcriptional control,
it is becoming increasingly clear that the early stages of SR signaling also contribute to
the complexity of steroidal responses. A nexus of this complexity occurs when hormone-
free SRs enter into large heteromeric complexes containing the molecular chaperone
HSP90 and the co-chaperone p23 (Pratt and Toft, 1997). Almost all members of the SR
family have been shown to contain both of these components. However, a number of
additional co-chaperones have been identified that seem to variably enter into hormone-
free SR complexes (Pratt et al., 2004b; Smith, 2004). These are FK506-binding protein
52 (FKBP52), the closely related FK506-binding protein 51 (FKBP51), cyclosporin A-
binding protein (Cyp40) and protein phosphatase 5 (PP5). A common feature of these
proteins is the presence of imperfect tetratricopeptide repeat (TPR) motifs that serve as
protein-protein interaction domains (Goebl and Yanagida, 1991). Indeed, TPR proteins
enter into SR complexes through a direct binding to HSP90 at its C-terminal TPR
acceptor site (Radanyi et al., 1994; Russell et al., 1999; Scheufler et al., 2000).

Although the roles played by HSP90 to control SR function are fairly well
understood (Pratt et al., 2004a), control of SR activity by TPR proteins remains largely
unresolved. One reason for this is that the TPR acceptor site of HSP90 can accommodate only one TPR protein at a time (Renoir et al., 1995; Carrello et al., 1999; Silverstein et al., 1999). This fact means that several distinct SR heterocomplexes are possible, even in the same cell, based on TPR protein composition. Thus, many reports of SR interactions with FKBP51, FKBP52, Cyp40 and PP5 have come forward, and it has become the conventional wisdom that all SRs can and do interact with all four of these TPRs. According to this school of thought, the four TPRs regulate distinct, but as yet undefined, stages of the signal pathway common to all receptors. Yet, a competing school of thought exists. It holds that receptors can be preferentially regulated by one TPR over another. Although there is less evidence in support of the latter model, we believe this is so because proper investigation of this concept requires analysis of multiple TPRs across multiple receptor types.

Since the early studies of Jensen and Gorski (Noteboom and Gorski, 1965; Jensen et al., 1968), a central issue has been the location within the cell of hormone-free SR complexes. Because investigations with the first SR antibodies were done on estrogen and progesterone receptors (PR) (Greene et al., 1984; Perrot-Applanat et al., 1985), it became early dogma that all SR family members reside in the nucleus, even in the absence of hormone. Thus, it was met with some skepticism when investigations on glucocorticoid (GR), mineralocorticoid (MR) and androgen receptors (AR) showed localization of these receptors to the cytoplasm (Wikstrom et al., 1987; Lombes et al., 1990; Georget et al., 1997; Fejes-Toth et al., 1998). Although this distinction within the SR family is now widely accepted as fact, the underlying mechanism that differentially
controls SR location remains unresolved. Some advances, however, have been made. It is now clear that cytoplasmic localization of GR, for example, is not an artifact or idiosyncrasy of cell types, as GR can localize to the cytoplasm in the very same cells where estrogen receptor or PR are localized to the nucleus (Picard et al., 1990; Wan et al., 2001). More importantly, we now know that SRs control their own localization through functional regions residing within the hinge and ligand-binding domains (LBD). Early work by Yamamoto and colleagues identified the presence of two nuclear localization signals, NLS1 and NLS2, on the GR (Picard and Yamamoto, 1987). When isolated from the rest of the GR polypeptide, NLS1 caused constitutive nuclear localization, whereas the larger and poorly defined NLS2 region affected nuclear translocation only in response to hormone. Because both PR (Guiochon-Mantel et al., 1989; Ylikomi et al., 1992) and GR contain homologous NLS1 domains, it is unlikely that NLS1 serves as the basis for differential localization of the hormone-free receptors. Although it has been postulated that the NLS1 of PR may be masked either by its own LBD or by association with HSP90, we believe more recent work by Nordeen and colleagues (Wan et al., 2001) provides a better working model for understanding localization of SRs under hormone-free conditions. By comparing chimeric forms of GR and PR, the Nordeen group identified a common region within the LBD of each receptor that imparts specificity to cellular localization of hormone-free receptors. This region, therefore, may represent a third and more general cellular localization signal, or CLS.

The CLS domain encompasses helices 1 through 5 at the N-terminal end of the LBDs of both GR and PR (Wan et al., 2001). Interestingly, this region coincides with the
putative signal transduction domain originally proposed by Pratt and colleagues (Pratt et al., 1988), which serves as a principal site for the SR/HSP90 interaction (Scherrer et al., 1993). Because HSP90 is a mediator for TPR protein binding, and because FKBP52 is known to bind the motor protein dynein (Czar et al., 1994; Silverstein et al., 1999), involvement of the HSP90/FKBP52 complex in SR trafficking has been proposed. Evidence in support of this role for FKBP52 includes the inhibition of “hormone-induced” GR translocation by over-expression of the FKBP52 dynein-interaction domain (Galigniana et al., 2001). Interestingly, the Rein laboratory found that FKBP51, in contrast to FKBP52, had an inhibitory role on GR translocation (Wochnik et al., 2005), suggesting that these two closely related FKBPs have antagonistic effects on hormone-driven GR movement. Work from our laboratory supports this conclusion. The GR of L929 cells is found in the cytoplasm prior to hormone binding as a complex containing FKBP51. Addition of hormone caused FKBP51 to be exchanged for FKBP52, generating a transient complex that localized to the nucleus (Davies et al., 2002; Davies et al., 2005). Taken as a whole, these data are further support for an active role of FKBP52 in “hormone-induced” GR translocation. The data also suggests the intriguing possibility that differential incorporation of TPRs, such as FKBP51 and FKBP52, into steroid-free receptor complexes could form the basis for known differences in receptor localization.

In this work, we address this hypothesis by determining the TPR protein composition of hormone-free GR and PR heterocomplexes and the contribution of the TPRs (FKBP51, FKBP52, PP5 and Cyp40) to differential localization of the receptors. We show that GR and PR expressed in the same cell localize to the cytoplasm and
nucleus, respectively, and that each receptor has distinct preferences for TPR proteins—GR recruits FKBP51 and PP5 complexes, while PR recruits FKBP52. We also show that TPR specificity is controlled by the receptor LBD, and that TPR expression levels can alter, to a degree, both the TPR composition of the receptors and their localization. Thus, our work may provide the missing connection between the known ability of steroid receptors to control their own localization and the potential factors that mediate the process.

RESULTS

As already mentioned, the GR of mouse L929 fibroblast cells is primarily found in the cytoplasm. Interestingly, the distribution of mouse GR expressed in Chinese hamster ovary cells (e.g., WCL2 cells) is shifted to the nucleus (Sanchez et al., 1990a), although no explanation for this discrepancy has been found. In COS cells, GR is once again found in the cytoplasm, but PR expressed in the same cells is almost exclusively nuclear (Wan et al., 2001). Although the Nordeen laboratory showed that control of localization in the COS system resides within the ligand-binding domain of each receptor, the factors responsible for the differential localization have yet to be discovered. Here, we exploit these differences to test the hypothesis that differential recruitment of TPR proteins may form the basis for differential localization of hormone-free steroid receptor complexes.
Expression Profile and Subcellular Localization of TPR proteins in L929, WCL2 and COS-1 Cells

For reasons described above, we have chosen L929, WCL2 and COS-1 cells to pursue the goals of this project. As a first step, we assessed the relative expression levels of FKBP51, FKBP52, PP5 and Cyp40 in these cells (Fig. 1). At the outset, it should be noted that the quantitative immunoblot procedure used throughout this work cannot measure true stoichiometry, but can be used to determine ratios between different proteins in the same cell line or between the same protein in two or more cell types. The results show that WCL2 cells have more FKBP52 than L929 and COS-1 cells, while levels of FKBP51 are the highest in L929. Levels of Cyp40 and PP5 are comparable in WCL2 and COS-1 and are considerably less in L929 cells.

We next examined the subcellular localization of these proteins in each cell line, as this could potentially affect receptor localization. Detection of each TPR was achieved through indirect immunofluorescence (Fig 2). In L929 and WCL2 cells, FKBP51 appeared to have about equal distribution between the cytoplasm and nucleus. In contrast, the FKBP51 signal in COS-1 cells was strongest in the nucleus. It is widely accepted that COS-1 cells, due to their distended cytoplasmic morphology, are better suited for analysis by subcellular imaging. Thus, it is tempting to make conclusions of distribution based solely on the COS-1 results. However, there is no guarantee that TPR distribution will be the same across all cells lines, as the results with FKBP51 imply. Distribution of FKBP52 was very similar in the three cell lines, in each case showing a strong cytoplasmic signal with some nuclear content. In COS-1 cells, the FKBP52 signal showed a clear
localization to the cytoskeletal network, a result consistent with earlier publications
demonstrating co-localization of FKBP52 to microtubule filaments (Ruff et al., 1992;
Czar et al., 1995) and the ability of FKBP52 to bind the motor protein dynein (Silverstein
et al., 1999). The intracellular patterns of PP5 and Cyp40 were highly similar, each
showing distribution mostly to the cytoplasm, with a small fraction in the nucleus.

**Differential Localization and Recruitment of TPR Proteins by GR in L929 and
WCL2 Cells**

As mentioned above, GR in L929 cells is reported to be cytoplasmic under
hormone-free conditions, while mouse GR in WCL2 is primarily found in the nucleus
(Sanchez et al., 1990a). As seen in Fig. 3A, we have confirmed these properties through
use of indirect immunofluorescence. The results show that GR of L929 was found in both
cellular compartments, while WCL2 GR was found primarily in the nucleus. In both cell
lines, addition of dexamethasone caused a coalescence of the GR signal within the
nucleus. Interestingly, although hormone-free GR of WCL2 cells was nuclear, it is not
tightly bound to this compartment and can be released into the cytosolic fraction upon
cell rupture (Fig. 3B). Thus, the GR of WCL2 cells most likely exists as a nuclear
heterocomplex containing HSP90 and one or more, as yet undefined, TPR proteins.

We have shown that the GR of L929 cells is cytoplasmic with FKBP51 as the
major TPR and that binding of hormone causes a shift of GR to the nucleus as a complex
containing FKBP52 (Davies et al., 2002). We consequently speculated that FKBP52
recruitment to receptor complexes might be required for nuclear localization. It was
therefore interesting that WCL2 cells were found to have higher levels of FKBP52 than
L929 cells (Fig. 1), suggesting that recruitment of FKBP52 in place of FKBP51, due to naturally-occurring FKBP52 over-expression, could lead to altered localization of GR. To test this hypothesis, we determined the FKBP composition of unliganded GR heterocomplexes in L929 and WCL2 cells (Fig. 4). Because other TPRs could also be involved in localization, we determined the status of PP5 and Cyp40 in the GR complexes. Interestingly, the amount of PP5 recruited by GR was about the same in both cell types, suggesting that PP5 is not a likely determinant of localization for this receptor. Cyp40 was not found to enter the receptor complex in either cell type. However, a clear distinction was found when comparing FKBP results. As expected, GR in L929 cells contained FKBP51 with little or no FKBP52, but this ratio was reversed for the WCL2 GR.

**Over-expression of FKBP51 but not FKBP52 Alters GR Localization**

In Fig. 1, we showed that WCL2 cells expressed a higher ratio of FKBP52 to FKBP51 than did L929 cells, where the ratio was essentially reversed. Thus, the differential interaction of GR with FKBP51 and FKBP52 seen in L929 and WCL2 cells (Fig. 4) suggests a model in which the equilibrium ratio of FKBP5s is a major determinant of GR interaction and localization. To test this model, we performed a series of over-expression experiments using FLAG-tagged FKBP51 and FKBP52 (Fig. 5). In the first, FLAG-tagged FKBP51 was over-expressed in WCL2 cells. Co-immunoprecipitation analysis showed recruitment of newly expressed FKBP51 to the WCL2 GR complex (Fig. 5B). Befitting the model, GR localization was also changed upon FKBP51 over-expression, as GR was no longer confined to the nuclear compartment but showed at least
a partial shift to the cytoplasm (Fig. 5E). We next over-expressed FLAG-tagged FKBP52 in L929 cells. Interestingly, recruitment of FLAG-FKBP52 to the GR could not be detected using antibodies to theFLAG epitope (Fig 5C) or FKBP52 itself (Fig. 5D), even though levels of FLAG-FKBP52 expression in the L929 cells were comparable to that achieved for FLAG-FKBP51 in the WCL2 cells (Fig. 5A). Because FLAG-FKBP52 failed to interact with GR, it was not surprising that GR localization was unchanged following transfection (Fig. 5E). Based on these results, it appears that the equilibrium model cannot be the only process controlling FKBP interaction with GR. Indeed, the facility by which GR of WCL2 cells interacted with FLAG-FKBP51 and the native interaction of L929 cell GR with the same TPR, both point to a preference of hormone-free GR for the FKBP51 over FKBP52.

**Expression of GR, PRB and Chimeras in COS-1 Cells**

If GR has a distinct TPR specificity, a further test of this model would be to ask if TPR specificity is applicable to other steroid receptors, such as PR. We chose to work with PRB, which is predominantly nuclear in most cells (Wikstrom et al., 1987; Lim et al., 1999) and compare its TPR specificity and localization to GR. To achieve this, wild-type (WT) and chimeric forms of GR and PRB were obtained (a generous gift of Steve Nordeen Ph.D) which have green fluorescent protein (GFP) fused to the N-terminal domain (NTD) of each receptor (Fig. 6A). The chimeras of GR and PRB were constructed so as to achieve exchange of ligand-binding domains (LBD). Thus, the GR/PR-LBD chimera has the entire N terminus, DNA-binding domain (DBD) and hinge region (H) of hGR linked to the LBD of hPRB. The PR/GR-LBD is constructed in an
analogous manner. Expression vectors encoding the GR, PR, GR/PR-LBD and PR/GR-LBD fusion proteins were transiently transfected into receptor-less COS-1 cells so as to achieve comparable levels of expression (Fig. 6B).

**TPR Specificity Correlates with Localization and Maps to the Ligand-binding Domain**

Using the GR and PR constructs described above, Wan et al. (2001) showed that differential intracellular localization of receptors was controlled by the ligand-binding domain (Wan et al., 2001). To determine the role played by TPRs in this process, we first confirmed the localization properties of these receptors (Fig. 7A). In COS-1 cells, unliganded GR was predominantly cytoplasmic and became completely nuclear in the presence of dexamethasone. As also expected, hormone-bound GR did not localize to nucleoli. In contrast, PRB was mostly nuclear in the absence of the progestin agonist (R5020), although a small portion was observed in the cytoplasm, as previously reported (Perrot-Applanat et al., 1986; Lim et al., 1999). In the presence of R5020, PRB became strictly nuclear but was also excluded from nucleoli. Analysis of the GR/PR-LBD and PR/GR-LBD chimeras confirmed that differential localization of GR and PR is indeed determined by the LBD of each receptor.

To identify the TPRs specific to each receptor construct, co-immunoprecipitation analysis was performed (Fig. 7B). Like GR in L929 cells, wild-type GR in the COS-1 cells was found to associate with FKBP51 and PP5, but no Cyp40 or FKBP52. Thus, the selectivity of GR for both of these TPRs appears to hold true in the COS cell type. Because wild-type PR had a strong nuclear signal, we expected to see an association with
FKBP52. This was found, but interaction with FKBP51 was also observed, suggesting that the fraction of PR found in the cytoplasm may interact with this TPR. Most interestingly, co-immunoprecipitation analysis of the chimeras showed that TPR specificity is determined by the LBD. Thus, the GR/PR-LBD chimera, like wild-type PR, was found to bind FKBP52 and FKBP51, whereas PR/GR-LBD interacted with FKBP51 and PP5. Although both GR and PR interact with FKBP51, the wild-type GR and PR/GR-LBD constructs resulted in a higher yield of FKBP51. These results not only reaffirm the selectivity of GR for FKBP51, but also for the first time show that the ligand-binding domain is the site of this specificity.

**Analysis in Wild-type and TPR-deficient Mouse Embryonic Fibroblast Cells**

Based on our co-immunoprecipitation results in COS cells, FKBP51 and PP5 were the only TPRs interacting with GR while, FKBP52 and smaller amounts of FKBP51 were specific for PRB. Therefore, we set out to determine how localization of GR and PRB would be impacted in the absence of FKBP51, FKBP52 or PP5. To address this question, we chose to work with a set of TPR-deficient mouse embryonic fibroblast (MEF) cells generated from mice with targeted ablation of each gene. Our first descriptions of altered physiological responses in FKBP52 KO and FKBP51 KO animals have been described elsewhere (Yang et al., 2006; Yong et al., 2007). As seen in Fig. 8A, each KO MEF cell line is completely devoid of its respective TPR. We also performed co-immunoprecipitation analysis of GR complexes expressed in WT MEF cells (Fig. 8B). The results were consistent with those obtained in the COS cells, showing GR specificity.
for FKBP51 and PP5. Thus, analysis of GR localization in the TPR-deficient MEF cells should not be complicated by cell-type specific TPR properties.

**Altered Localization of GR and PRB in FKBP51 KO MEF Cells**

To determine how absence of FKBP51 might affect localization of receptors, GFP-GR and GFP-PRB were transfected into WT and FKBP51 KO MEF cells. Confocal microscopy was conducted on live, unfixed cells to determine localization under hormone-free and hormone-bound conditions (Fig. 9A). As expected, GFP-GR in WT MEF cells was found exclusively in the cytoplasm. In FKBP51 KO MEFs, however, a fraction of unliganded GR shifted to the nucleus. In both WT and FKBP51 KO MEF cells, the entire GFP-GR population translocated to the nucleus when hormone was added. Altered localization was also observed for GFP-PR. In WT MEF cells, the PRB signal was strongest in the nucleus, but a distinct subpopulation could be found in the cytoplasm. Thus, PR and GR have consistent and receptor-specific patterns of localization in the COS and MEF cell types. In FKBP51 KO cells, the cytoplasmic PR signal was drastically reduced, suggesting that FKBP51 determines the cytoplasmic localization of a subpopulation of PR. Figure 9B shows magnified views of the same experiment performed on a different set of WT and FKBP51 KO cells.

**FKBP52 Deficiency Only Affects PRB Localization**

In Fig. 10, GFP-GR and GFP-PRB were transfected into WT and FKBP52 KO MEF cells and subjected to confocal microscopy. The results suggest that FKBP52 does not play a crucial role in the localization of GR, as WT and FKBP52 KO MEFs had
similar distribution patterns for GR: cytoplasmic in the absence of hormone and completely nuclear in its presence. The hormone-free results were not surprising since the GR complex of MEF cells, like those of L929 and COS cells, does not interact with FKBP52 (Fig. 8). What was surprising is that “hormone-induced” translocation of GR appeared to be normal in the FKBP52 KO cells, given our prior observation that “hormone-induced” swapping of FKBP52 for FKBP51 correlated with nuclear translocation of GR (Davies et al., 2002). [See below for further discussion of this issue.] In the case of PR, loss of FKBP52 did have a major impact on localization. Hormone-free PRB became almost completely cytoplasmic in FKBP52 KO cells, mimicking the localization of GR. Interestingly, the cytoplasmic PR of FKBP52 KO cells readily translocated to the nucleus in response to hormone. Thus, like GR, PRB movement from cytoplasm to nucleus was also unaffected by FKBP52 loss.

**No Effect of PP5 Loss on GR or PRB Localization**

Little direct or circumstantial evidence exists for a role of PP5 in control of steroid receptor localization. One exception to this is a report from Pratt and colleagues showing an interaction between PP5 and the motor protein, dynein (Galigniana et al., 2002). This would suggest that PP5 could be serving a compensatory role in the FKBP52 KO cells, allowing GR to translocate in response to hormone. Yet, PP5 is found in all GR complexes analyzed in this work, most of which are present in the cytoplasm, but one of which (WCL2 GR) is present in the nucleus. Thus, it is more likely that PP5 is either neutral with respect to GR localization, or that it is a contributor to localization in the cytoplasm. The latter hypothesis is supported by a report from the Honkanen laboratory...
demonstrating a shift of hormone-free GR to the nucleus following RNAi down-regulation of PP5 (Dean et al., 2001). To address this controversy, GR and PRB localization was determined in PP5 KO MEF cells (Fig. 11). Distribution of GR in PP5 KO was very similar to WT, with the majority of receptor confined to the cytoplasm and showing nuclear translocation in response to dexamethasone. Thus, even though GR complexes contain PP5, it does not appear to play a significant role in the localization properties of this receptor. With respect to PRB, we did not expect PP5 to play a decisive role in its localization, since PP5 was never found in PRB complexes. Our findings in the MEF cells are in accordance with this prediction, as PRB distribution was the same in WT and PP5 KO MEF cells. Moreover, addition of R5020 resulted in nuclear translocation of the cytoplasmic subpopulation of PRB, indicating normal functioning of this process.

**DISCUSSION**

In this work, we tested the hypothesis that the ability of hormone-free steroid receptors to reside in distinct cellular compartments is mediated by differential interactions with TPR proteins. Based on our results, we are able to make several conclusions. First, it is now clear that steroid receptors, as exemplified by GR and PRB, have specific preferences for TPR proteins. With the exception of GR in WCL2 cells, GR found in L929, COS and MEF cells all entered into complexes containing FKBP51 and PP5. In the case of PRB, a distinct preference for FKBP52 and, to a lesser degree, FKBP51, was observed in COS cells. Second, receptor specificity for TPR composition resides in the ligand-binding domain – the same domain that determines receptor localization. Third, receptor
localization is controlled by TPR recruitment, especially the presence or absence of FKBP51 and FKBP52 in the complexes. This last conclusion is based on the facts that recruitment of FKBP52 always correlated with nuclear localization (PRB in MEF and COS cells, GR in WCL2 cells), while recruitment of FKBP51 correlated with receptor localization to the cytoplasm (GR of L929, COS and MEF cells). Moreover, alteration of FKBP51 or FKBP52 protein levels through over-expression or targeted ablation caused receptor localization, in most cases, to also change. Thus, TPR specificity appears to be causative with respect to localization.

Perhaps the most interesting new question posed by our data is how TPR specificity encoded in the receptor LBD gets transduced into differential recruitment of TPR proteins. Because the TPR interaction occurs through HSP90, it is possible that some type of inter-molecular cross-talk may be occurring, with HSP90 as transducer. According to this model, the SR/HSP90 interaction would likely have to cause specific and distinct alterations to HSP90 conformation at the TPR acceptor site, leading to an exchange reaction for TPRs. Our prior demonstration that the hormone-binding event can cause GR to undergo an exchange of FKBP51 for FKBP52 (Davies et al., 2002) is evidence in support of this model. Interestingly, support for inter-molecular cross-talk also exists in the opposite direction. For example, we found that treatment of L929 cells with FK506 increased the hormone-binding affinity of GR by a mechanism involving displacement of FKBP51 and increased recruitment of PP5 (Davies et al., 2005). Similar work by the Scammell laboratory has shown that elevated incorporation of FKBP51, especially the variant found in Squirrel monkeys, into GR complexes leads to reduced
hormone-binding affinity (Reynolds et al., 1999; Denny et al., 2000). Thus, if TPRs can control SR hormone-binding function, presumably through HSP90 mediation, then SR control of TPR specificity is not only reasonable but is likely to occur through the same mechanism. Of course, a caveat that must be considered is that TPR selectivity by receptors may occur at the level of HSP90 – namely, that SRs select distinct HSP90 complexes that differ with respect to TPR protein composition.

Most of our data are consistent with TPR specificities in which GR prefers FKBP51 and PP5, while PRB prefers FKBP52 and, to a lesser degree, FKBP51. A notable exception, however, was the GR of WCL2 cells, which were found predominantly in the nucleus and which showed a distinct preference for FKBP52 over FKBP51. In this respect, the WCL2 cell GR behaves very much like PRB and, therefore, provides further support for the notion that FKBP51 and FKBP52 provide “ying-yang” control over cellular localization of hormone-free SRs. Because WCL2 cells were found to express more FKBP52 and less FKBP51 than L929 cells, it is reasonable to assume that expression levels of TPRs can override specificity. Indeed, when FKBP51 was over-expressed in the WCL2 cells, incorporation of FKBP51 into GR complexes and a partial redistribution to the cytoplasm was seen. However, we were not able incorporate over-expressed FKBP52 into the GR complex of L929 cells, nor change its localization. Although it is possible that we did not sufficiently alter the FKBP51/FKBP52 ratio in the L929 cells, it is also possible that cell-type specific factors may be at play that imposes stringent controls on the SR/TPR interaction. This may explain why certain cells, such as human lymphocytes, have GR complexes with a high FKBP52 content (Reynolds et al.,
1999). Going forward, this will be an important issue to resolve, especially at the level of physiology, as tissue-specific control of TPR association within and across receptor types may be an important regulatory mechanism.

To test whether TPR proteins played active roles in the localization of receptors, we used a series of MEF cells completely deficient in FKBP51, FKBP52 or PP5. The results obtained for PRB were fairly dramatic. For example, loss of FKBP51 caused the cytoplasmic PR signal of WT cells to coalesce to the nucleus, providing strong evidence that a sub-population of PRB exists which resides in the cytoplasm based on its interaction with FKBP51. Conversely, loss of FKBP52 caused a redistribution of PRB to the cytoplasm, showing that its association with FKBP52 controls the strong nuclear localization of most PRB. In contrast, results with GR were less dramatic. No change in GR localization was seen in the FKBP52 KO cells. However, this was not surprising, as GR in WT MEF cells does not interact with FKBP52. Curiously, GR in the FKBP51 KO cells did not completely re-localize to the nucleus. Instead, only a fraction did so. We propose that one of two mechanisms may account for this result. First, because GR also interacts with PP5 and because we do not yet know the stoichiometry of PP5-containing to FKBP51-containing GR complexes in these cells, it may simply be that only a fraction of GR was affected by FKBP51 loss. Second, PP5 may have the ability to compensate, at least partially, for the loss of FKBP51. Indeed, evidence for this already exists, as use of FK506 to remove FKBP51 from the GR complexes of L929 cells causes PP5 to take its place (Davies et al., 2005). For these reasons, it was important to test GR localization in the PP5 KO cells. Yet, PP5 loss had no effect on GR. Based solely on this result; it is
tempting to conclude that PP5 does not control GR localization. However, this conclusion may be premature, as compensation by FKBP51 for loss of PP5 may also be occurring. Clearly, the compensation model is an important issue in this field that will require new advancements, such as the generation of cells with dual deficiency of FKBP51 and PP5, as well as cells with other combinations of missing TPRs.

Because there is substantial literature suggesting that both HSP90 and FKBP52 contribute to “hormone-induced” translocation of GR to the nucleus (Czar et al., 1994; Galigniana et al., 1998; Galigniana et al., 2001; Davies et al., 2002; Galigniana et al., 2004a; Wochnik et al., 2005), our finding of “normal” translocation by GR in the FKBP52 KO cells could be seen as a repudiation of this concept. However, two issues should be kept in mind when evaluating our data. First, in the studies cited above, the data support a role for HSP90 and FKBP52 only rapid, microtubule-based transport of GR. For reasons that are still unknown, passive diffusion of hormone-bound GR to the nucleus can also occur, even in the complete absence of cytoskeleton (Galigniana et al., 1998). In this work, we used a hormone treatment time of 1 h—a timepoint that does not allow discrimination between active transport and passive diffusion. Therefore, the only conclusion we can safely make is that GR of FKBP52 KO cells can still respond to hormone and that the overall, long-term translocation response is not affected. Second, because there is evidence that both Cyp40 and PP5 can also interact with dynein (Galigniana et al., 2002); it is possible that one or both of these proteins can compensate for the loss of FKBP52. Thus, a vigorous investigation of TPR involvement in GR
translocation will require both kinetic analysis and the use of cells with various combinations of dual TPR deficiency.

As mentioned earlier, we have published our first descriptions of altered physiology in FKBP52 KO and FKBP51 KO mice (Yang et al., 2006; Yong et al., 2007). Although the present work provides data that corroborates the physiological studies, other aspects of our new data do not – at least, not without further investigation. For example, here we show that the principal TPRs interacting with GR are FKBP51 and PP5. Therefore, it is not surprising that FKBP52 KO mice show no dramatic alterations to GR-controlled physiology. However, the FKBP51 KO mice also appear normal with respect to GR. A possible explanation for both observations could be mutual compensation between FKBP51 and FKBP52 for GR functionality. To test this, compound FKBP51/FKBP52 KO animals were also generated (Yong et al., 2007). Unfortunately, this genotype is an early embryonic lethal, making further investigation difficult. Going forward, we plan to focus on the FKBP51 KO animals, utilizing stress and metabolic challenges to uncover potential alterations to GR responses.

With respect to PRB, here we show a major interaction with FKBP52 and minor interaction with FKBP51. Befitting this data, FKBP51 KO females show no obvious alterations to PR-regulated responses, such as fertility. Interestingly, FKBP52 KO females are sterile; principally due to a failure of uterine receptivity to implantation (Yang et al., 2006). Reduced ovulation and mammary gland ductal development also occurs in FKBP52 KO females. Based on the PRAKO/PRBKO studies of Conneely and colleagues (Lydon et al., 1995; Mulac-Jericevic et al., 2000; Mulac-Jericevic et al.,...
2003), those data suggest that loss of FKBP52 has a moderate effect on PRB functionality (ovary, mammary) but a more dramatic effect on PRA (uterus). Indeed, comparison of uterine PRA and PRB showed a greater interaction of the A isoform with FKBP52. It will therefore be interesting to determine if PRA, like PRB, interacts, at least in part, with FKBP51, or whether it is exclusively regulated by FKBP52. The latter outcome may therefore explain the relative impact of FKBP52 loss on the two PR isoforms, as compensation by FKBP51 may occur for PRB but not PRA.

On a greater note, it is differences like those observed for PRA versus PRB that underscore the importance of precisely defining the TPR interactions for each receptor. Indeed, we propose that a proper understanding of TPR proteins and the roles they play in steroidal responses will require the methodical and systematic characterization of the distinct SR/TPR interactions that are likely to occur across cell lines and tissues. Such an atlas should prove invaluable to the development of new strategies and therapeutics to combat endocrine disorders.

MATERIALS AND METHODS

**Materials** - Dexamethasone, R5020, methotrexate, dextran, HEPES, DMEM powdered medium, protein A-Sepharose, protein G-Sepharose, Tris, EDTA, PBS, N, N, N', N'-tetramethylenediamine, sodium molybdate, protease inhibitor cocktail, non-immune mouse IgG2A, goat anti-mouse IgG-horseradish peroxidase conjugate, sodium chloride, luminol, coumaric acid, hydrogen peroxide (30% w/w), anti-flag M2 monoclonal IgG (F-3165) and laminin were all obtained from Sigma (St. Louis, MO). Iron-supplemented
newborn calf serum was from Hyclone Laboratories Inc. (Logan, UT). Immobilon polyvinylidene fluoride (PVDF) membrane was obtained from Millipore Corporation (Bedford, MA). Lifefectamine 2000 transfection reagent, OPTI-MEM, glycerol and goat serum used for blocking in immunofluorescence were obtained from Invitrogen Corporation (Carlsbad, CA). FiGR monoclonal antibodies against GR and rabbit polyclonal antibody against PP5 were generous gifts from Jack Bodwell (Dartmouth Medical School, Hanover, NH) and Michael Chinkers (University of South Alabama, College of Medicine, Mobile, AL) respectively. Antibodies against FKBP51 (sc-11518), FKBP52 (sc-1803), bovine anti-goat IgG-HRP conjugate (sc-2350) and anti-Green Fluorescent Protein (GFP) mouse monoclonal IgG (sc-9996) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibody against Cyp40 (PA3-022) was purchased from Affinity Bioreagents (Golden, CO). Goat anti-rabbit IgG-HRP conjugate (401315) and fluorescein-conjugated goat anti-mouse IgG (401234) was purchased from Calbiochem (LA Jolla, CA). Fluorescein-conjugated donkey anti-rabbit IgG (A-21206) was purchased from Molecular Probes Inc. (Eugene, OR). Fluorescent mounting medium was purchased from Dakocytomation (Carpinteria, CA).

**Cell Lines and Culture** - The L929 cells were routinely cultured and maintained in Dulbecco’s Modified Eagles’s Medium (DMEM) containing 10% iron-supplemented newborn calf serum. The WCL2 cells (Hirst et al., 1990) are derivatives of CHO cells subjected to rounds of methotrexate amplification after co-transfection with plasmids containing the cDNA for mouse wild-type GR and dihydrofolate reductase. WCL2 cells
were maintained in 10µM methotrexate. COS-1 cells (African green monkey kidney cell line, a generous gift from Brian Rowan) were maintained using DMEM containing 10% iron-supplemented newborn bovine calf serum. Mouse embryonic fibroblasts (MEF) were isolated from wild-type, FKBP52 KO, FKBP51 KO and PP5 KO E13.5 embryos. Cells were cultured in DMEM with 15% FBS until confluence. Fibroblasts were the only cells that attached and proliferated. Immortalized MEF were generated by transfecting primary cells with vector for SV40-large T antigen. Transformed cells maintain normal MEF morphology with high proliferative activity.

**Transient Transfection**- For transient transfection, cells were plated on a 60mm dish in DMEM containing 10% iron-supplemented calf-serum pre-stripped of endogenous steroids by 1% (w/v) dextran-coated charcoal for 24 h prior to transfection and allowed to grow to 85-90% confluency. Cells were washed with OPTI-MEM and transfected using Lipofectamine 2000, according manufacturer’s protocol. OPTI-MEM was removed after 5h and DMEM containing dextran-charcoal stripped serum was added. All hormone treatments were done 16-20 h post-transfection for 1 h.

**Whole Cell Extraction**- Cells were washed and collected in 1X PBS followed by centrifugation at 1520 X g for 10 min. The supernatant was discarded and the pellet was re-suspended in 1X PBS. After a short spin at 20,800 X g for 5 min at 4°C, the pellet was rapidly frozen on dry ice and stored at -70°C for 30 min. The frozen pellet was then re-suspended in 3 volumes of cold whole cell extract buffer (20mM HEPES, 25% glycerol,
0.42M NaCl, 0.2mMEDTA, pH 7.4) with protease inhibitors and incubated on ice for 10 min. The samples were centrifuged at 100,000 X g for 10 min at 4°C. Protein levels were estimated spectrophotometrically by the BCA™ protein assay (Pierce). The supernatants were either stored at –80°C or used immediately for immunoblot analysis to determine protein expression levels.

**Immunoadsorption of GR and PR Complexes**- Cells were ruptured by Dounce homogenization in HEMG buffer (10 mM HEPES, 3 mM EDTA, 20 mM sodium molybdate, 5% glycerol, pH 7.4). Lysates were centrifuged at 20,000 X g for 30 min to generate cytosolic fractions (cytosol). All cytosols were used without freezing or storage. Immunoadsorption was performed by adding 10 µl of FiGR monoclonal against GR or non-immune mouse IgG2A to aliquots of cytosol along with 50µl of protein A-Sepharose. Samples were rotated at 4°C overnight. Pellets were washed five to six times with TEGM (10 mM TES, 3 mM EDTA, 10% w/v glycerol, 50 mM NaCl, 20 mM sodium molybdate, pH 7.4) followed by elution of receptor heterocomplexes with 2X SDS sample buffer. For all immunoadsorptions performed in COS-1 cells after transient transfection of GFP-tagged receptors, 10 µl of anti-GFP mouse monoclonal IgG or control antibody was added to the cytosolic extract along with 50 µl of protein G-Sepharose. G-instead of A-Sepharose was used to minimize non-specific binding by the GFP moiety.
**Gel Electrophoresis and Immuno Blotting**- Samples were resolved on denaturing SDS gels (Laemmli, 1970) using a 7-14% acrylamide gradient in order to achieve maximal separation between the immunophilins and antibody heavy chains. Transfer of the samples to Immobilon membranes and quantitative immunoblotting were then performed. The FiGR antibody against GR was used to probe for receptor (Figs. 3, 4, 5 and 8), while various antibodies were used to probe for FKBP52 [sc-1803; Santa Cruz Biotechnologies], FKBP51 [sc-11518; Santa Cruz Biotechnologies], Cyp40 [PA3-022; Affinity Bioreagents], and PP5 [a gift from Michael Chinkers]. Anti-FLAG M2 monoclonal IgG [F-3165; Sigma] and anti-GFP mouse monoclonal IgG [sc-9996; Santa Cruz Biotechnologies] were used in the experiments of Figs. 5 to 7). After probing, blots were incubated with appropriate HRP-conjugated counter antibodies. Proteins were detected using luminol [A8511; Sigma], coumaric acid [C9008; Sigma] and hydrogen peroxide [H1009; Sigma] via enhanced chemiluminescence (ECL). Quantitation was performed using BIO-RAD GS-670 imaging densitometer (BIO-RAD)

**Indirect Immunofluorescence**- L929, WCL2 and COS-1 cells were seeded on laminin-coated cover slips in 60 mm dishes at 50,000 to 100,000 cells per dish. Forty-eight hours later cells, were washed with Hanks Blanced Salt Solution (HBSS) (pH7.4), fixed with ice-cold methanol for 10 minutes, and blocked for 30 min with 10% goat serum in PBS. Cells were then incubated with primary antibody for 1 hour in PBS with 10% goat serum. After three washes with 10% goat serum in PBS, the cells were then incubated for 1 h with either fluorescein-conjugated goat anti-mouse IgG at a dilution of 1:20 (401234, Cal
Biochem) or Alexa Fluor 488 goat anti-rabbit IgG at a dilution of 1:600 (A-21206, Molecular Probes) in PBS with 10% goat serum. Cells were once again washed 3 times with PBS and the cover slips were mounted onto slides with DAKO fluorescent mounting medium and sealed. Photomicrographs were taken with a Nikon Eclipse 800 fluorescence microscope equipped with a Sensys digital camera and Image Pro software (Media Cybernetics).

**Green Fluorescent Protein Imaging** - COS-1 and MEF cells were seeded on laminin-coated cover slips in 60mm dishes at 300,000 to 500,000 cells per dish. Cells were maintained in medium containing charcoal-stripped serum before fluorescence imaging. The cells were transfected 48 h later with either GFP-tagged GR and PRB constructs or empty vector (pEGFP-C1). Fluorescent images of the living cells were obtained 24 h post-transfection and 1 h after vehicle or hormone treatment using an Olympus IX70 inverted microscope equipped with a Leica DMIRE2 confocal microscope (Leica, Mannheim, Germany). Cells were scanned at lower laser power to avoid photo bleaching. Leica confocal software was used for data analysis. The figures show representative cells from each type of transfection. At least 50-100 cells from each transfection were inspected.

**ACKNOWLEDGEMENTS**

We wish to thank Dr. Jean Overmeyer (Department of Biochemistry and Cancer Biology, University of Toledo College of Medicine) for excellent advice in microscopy. We also thank Drs. Michael Chinkers (University of South Alabama) and Jack Bodwell
(Dartmouth University) for the gifts of PP5 and FiGR antibodies, respectively. We thank Drs. Steve Nordeen (University of Colorado) and Theo Rein (Max Planck Institute of Psychiatry) for the generous gifts of GFP constructs of steroid receptors and FLAG-tagged constructs of FKBP51 and FKBP52, respectively. This study was supported in part by National Institute of Health grants DK43867 (E.S.), DK70127 (E.S., W.S.), DK73402 (W.S., E.S), and the Riley Children’s Foundation (W.S.).

FIGURE LEGENDS

Fig. 1. Immuno Blot Profile of TPR proteins in L929, WCL2 and COS-1 Cells. (A) Expression levels of FKBP52, FKBP51, Cyp40, and PP5 were measured in whole cell lysates from L929, WCL2 and COS-1 cells. (B) Quantitation of TPR levels. Densitometric values were normalized as percent of WCL2 and represent the means ± SEM of three independent experiments.

Fig. 2. Comparison of TPR Intracellular Distribution. Indirect immunofluorescence was performed in L929, WCL2 and COS-1 cells, as described under Materials and Methods, using antibodies against FKBP51, FKBP52, PP5 and Cyp40. Results are representative of three independent experiments.

Fig. 3. Hormone-free GR in WCL2 Cells Is Loosely Bound In The Nucleus. (A) GR localization in L929 and WCL2 cells was detected by indirect immunofluorescence, as described under Materials and Methods. Cells were treated with vehicle or
dexamethasone (DEX, 1µM for 1 h), as indicated. (B) L929 and WCL2 cells treated with
or without dexamethasone (DEX, 1µM, 1 h) were fractionated into cytosolic (C) and
nuclear (N) extracts. Immunoabsorption was performed using FiGR antibody against GR,
followed by Western blotting. Results in (A) and (B) are representative of three
independent experiments.

Fig. 4. GR Complexes in WCL2 Cells Recruit Higher Levels of FKBP52 and Lower
Amounts of FKBP51 Compared to L929 Cells. (A) Analysis of TPR content in GR
heterocomplexes from L929 and WCL2 cells. Aliquots of L929 and WCL2 cytosol were
immunoabsorbed with FiGR antibody against GR or non-immune mouse IgG (NI).
Samples were split and analyzed by Western blotting with antibodies against GR, HSP90,
FKBP52, FKBP51, Cyp40, and PP5. (B) Quantitation of TPR protein levels in GR
heterocomplexes was accomplished by densitometric scanning of the films, followed by
subtraction of non-immune values and normalization to amount of GR protein in each
condition. Values represent the means ± SEM of three independent experiments. Cyp40
values are omitted due to lack of interaction with either GR.

Fig. 5. Over-expression of FKBP51 Alters GR Localization in WCL2 Cells, But No
Effect of FKBP52 on L929 Cell GR. (A) L929 and WCL2 cells were transiently
transfected with increasing amounts of FLAG-tagged FKBP52 or FLAG-tagged
FKBP51, respectively. Whole cell extracts were analyzed by Western blotting using anti-
FLAG mouse monoclonal antibody. (B) Analysis of FLAG 51 content in GR complexes
from WCL2 cells. WCL2 cells were mock transfected or transfected with FLAG-tagged FKBP51, followed by immunoadsorption of cytosols with FiGR antibody against GR or non-immune mouse IgG (NI). Samples were analyzed by Western blotting with antibody against GR or FLAG epitope. (C) Analysis of FLAG 52 content in GR complexes from L929 cells. L929 cells were transfected and analyzed as above, except FLAG-tagged FKBP52 was used. (D) Analysis of endogenous FKBP52 content in GR complexes from L929 cells. L929 cells were transfected and analyzed as in (C), except antibody against endogenous FKBP52 was used for blotting. Whole cell extracts (WCE) from wild-type (WT) FKBP52 KO MEF cells were used as controls. (E) Intracellular localization of GR in FLAG-FKBP51 or FLAG-FKBP52 transfected cells. Indirect immunofluorescence of GR in L929 and WCL2 cells following mock or FLAG-tagged transfection was performed as described under Materials and Methods. Results in (A – E) are representative of three independent experiments. In Panel (E), a minimum of 100 cells per condition was inspected.

Fig. 6. Domain Structure of GFP-tagged Wild-type and Chimeric Constructs of GR and PRB. (A) Schematic representation of wild-type human GR and human PRB linked to green fluorescent protein (GFP) at the N-terminal domain (NTD). Also shown are chimeric constructs in which the ligand-binding domains (LBD) of GR and PRB are exchanged. Helices 1-5 are principal regions of each LBD previously shown by Nordeen to control receptor localization in the absence of hormone (Wan et al., 2001). These helices coincide with the signal transduction domain (STD) previously shown by Pratt to
be a major site for HSP90 interaction (Pratt et al., 1988; Scherrer et al., 1993). DBD: DNA binding-domain; H: Hinge region. (B) Immuno blot profile confirming approximately equal expression of each construct in COS-1 cells. Lysates from COS-1 cells 48 h post-transfection were used for Western blot detection of GFP-tagged receptors with antibody against GFP.

**Fig. 7.** Ligand-binding Domains of GR and PRB Control Differential Localization and TPR Specificity. (A) LBD controls GR and PRB localization. COS-1 cells transfected with the indicated expression vectors were treated with vehicle, dexamethasone (100 nM), or R5020 (20 nM) for 1 h. In the case of chimeras, hormone selection was based on source of LBD. Fluorescence microscopy was performed on live, unfixed cells. (B) Recruitment of distinct TPRs by GR and PRB is controlled by the LBD. Cytosols were prepared from COS-1 cells transfected with wild type or chimeric receptor constructs. Equal aliquots of cytosol were used for immunoadsorption with antibody against GFP or non-immune mouse IgG (NI). Samples were split and analyzed by Western blotting with GFP antiserum to detect receptors, or antibodies against FKBP51, FKBP52, PP5 and Cyp40. Results in A and B are representative of three independent experiments. In Panel (A), a minimum of 100 cells per condition was inspected.

**Fig. 8.** Mouse Embryonic Fibroblasts Deficient in FKBP52, FKBP51 and PP5 and GR Complexes in WT MEFs. (A) MEF cells from mice with targeted ablation of FKBP52, FKBP51 or PP5 were made as described under Materials and Methods. Western blots
show expression levels of each TPR in the appropriate wild-type (WT) and TPR-deficient (KO) MEF cells. **(B)** TPR profile of GR heterocomplexes in WT MEF cells. Cytosols from WT MEF cells were immunoadsorbed with FiGR antibody against GR or with non-immune IgG (NI). Samples were split and analyzed by Western blotting for GR, HSP90, FKBP52, FKBP51, Cyp40 and PP5. Results in A and B are representative of two independent experiments.

**Fig. 9.** Deficiency of FKBP51 Causes Greater Nuclear Localization of GR and PRB. *(A)* Subcellular localization of GR and PRB in WT and FKBP51 KO MEF cells. MEF cells were transfected with GFP-GR or GFP-PRB followed by 1 h treatment with vehicle or dexamethasone (100 nM) or R5020 (20 nM). H: hormone-treatment. Confocal microscopy was conducted on live, unfixed cells. *(B)* Magnified view of typical distribution profile for each receptor in WT and FKBP51 KO MEF cells. Results in *(A)* and *(B)* are representative of 3 independent experiments. In Panel *(A)*, minimum of 100 cells per condition were inspected.

**Fig. 10.** Loss of FKBP52 Shifts PRB to the Cytoplasm, But Has No Effect on GR. *(A)* Subcellular localization of GR and PRB in WT and FKBP52 KO MEF cells. MEF cells were transfected with GFP-GR or GFP-PRB followed by 1 h treatment with vehicle or dexamethasone (100 nM) or R5020 (20 nM). H: hormone-treatment. Confocal microscopy was conducted on live, unfixed cells. *(B)* Magnified view of typical distribution profile for PRB in WT and FKBP52 KO MEF cells. Results in *(A)* and *(B)*
are representative of 3 independent experiments. In Panel (A), a minimum of 100 cells per condition was inspected.

Fig. 11. PP5 Deficiency Has No Effect on Localization of GR or PRB. WT and PP5 KO MEF cells were transfected with GFP-GR or GFP-PRB followed by 1 h treatment with vehicle or dexamethasone (100 nM) or R5020 (20 nM). H: hormone treatment. Confocal microscopy was conducted on live, unfixed cells. Results are representative of three independent experiments. A minimum of 100 cells per condition was inspected.
Fig. 1

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B

![Bar Graph Image]
Fig. 3

A

- DEX

+ DEX

L929  WCL2

B

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Fig. 5

A

DNA (µg): 0 2 8

FLAG 52

FLAG 51

L929

WCL2

B

WCL2

Mock Flag 51

IP: NI GR NI GR

Flag

GR

C

L929

Mock Flag 52

IP: NI GR NI GR

Flag

GR

NS

D

L929

Mock Flag 52 WCE

IP: NI GR NI GR 52KO 52WT

GR

NS

E

L929

MOCK

FLAG 52

WCL2

MOCK

FLAG 51

78
Fig. 6

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COS-1 Cells

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Fig. 7

A

Vehicle

Hormone

GR

PR

GR/PR-LBD

PR/GR-LBD

Vehicle

Hormone

B

NI  GFP  NI  GFP

PR

GR

FKBP51

FKBP52

HCP5

Cyp40

PR/GR-LBD

GR/PR-LBD

FKBP51

FKBP52

HCP5

Cyp40
Fig. 8

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Fig. 9

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FKBP51 KO MEF

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Fig. 10

A

WT MEF

FKBP52 KO MEF

B

WT MEF

KO MEF
Fig. 11
SUMMARY

The heterogeneity in the macromolecular structure of steroid receptors results from the presence of one of the many TPR domain proteins bound a single acceptor site located at the c-terminal end of the HSP90 dimer. FKBP51, FKBP52, Cyp40 and PP5 are four TPR proteins that compete for a single binding site on the HSP90 dimer of receptor complexes. Though several laboratories have published detailed reports documenting the role of these proteins in steroid receptor hormone-binding and transactivation function in yeast as well as mammalian cell lines, yet their role in determining receptor localization remains poorly understood. In this work, we test the differential control of TPRs on receptor localization. We chose a mouse fibroblast cell-line (L929), where unliganded GR is mainly cytoplasmic and a CHO-derived cell line (WCL2), which has over-expressed mouse GR predominantly nuclear in the absence of hormone.

To discriminate, we analyzed TPR protein levels by immuno blotting, receptor localization by immunofluorescence, and heterocomplex composition using co-IP assays. The differential recruitment of TPR protein by the GR complexes in WCL2 cells leading to altered sub cellular localization of the receptor correlated well with differences observed in the equilibrium levels of FKBP51 and FKBP52 in these cells compared to the L929 cells. We found a preferential recruitment of FKBP52 over FKBP51 by GR complexes in WCL2 cells, while the case was reversed in the L929 cells. Based on its ability to interact with motor protein dynein and primarily cytoplasmic distribution in WCL2 cells, FKBP52’s involvement in nuclear targeting of GR would be a reasonable speculation. On the other hand, it is more likely that by virtue of greater equilibrium
levels as well presence in the nucleus in case of the L929 cells, FKBP51 perhaps might play a significant role in the redistribution of GR to the cytoplasm. To ascertain, if altered GR localization was a consequence of limiting equilibrium concentrations of specific TPRs and if this situation could be reversed, we used FLAG-tagged 51 and 52 to alter the endogenous TPR levels. Expression of FLAG-tagged 51 in WCL2 cells led to redistribution of receptor complexes to the cytoplasm resulting from its recruitment into complexes, while, in L929 cells, expressing FLAG-tagged 52 receptor localization was not altered and GR remained primarily cytoplasmic. Thus, in WCL2 cells, specificity of GR for TPR protein FKBP51 remains unaffected even though its subcellular localization may be altered by cell-type-specific TPR expression. Based on the aforementioned findings receptor’s intrinsic affinity for FKBP51 does seem to predominate. However, it appears in the case of WCL2 cells that the preference of the receptor for FKBP51 can be out-competed by FKBP52 by a process not entirely determined by its relative abundance.

To further assess if distinct preferences for specific TPRs controlled subcellular localization of other steroid receptors as well, we expressed GFP-tagged wild and chimeric forms of GR and PR-B in receptor-less COS-1 cells. A series of co-immunoprecipitation assays identified differential TPR protein recruitment by both types of unliganded receptors to be responsible for the differences in localization observed by GFP fluorescence. In receptorless COS-1 cells, PR-B complexes recruited FKBP52 preferentially and were mainly localized in the nucleus while GR in COS-1 cells, like in the L929 cells, was mostly bound to FKBP51 and remained predominantly cytoplasmic.
in distribution. Using receptor chimeras, the LBD emerged as the site that controlled TPR protein specificity as well as subcellular localization.

Finally, to dissect the specific role of each of the TPR proteins, we compared localization of GR and PR-B under liganded and unliganded conditions in WT MEF to several KO MEF cell lines. In FKBP51 KO MEF, the normally cytoplasmic GR showed a greater nuclear distribution while in case of PR-B, the entire receptor population was almost completely nuclear in its distribution compared to the WT MEF cells. However, in FKBP52 KO cells, GR remained mostly cytoplasmic and the change in localization of PR-B was the most dramatic with almost the entire receptor population becoming cytoplasmic. Receptor translocation remained unaffected in FKBP51, PP5 and FKBP52 KO cells. In the case of GR, the receptor’s preference for FKBP51 and PP5 suggests the possibility of overlapping functions with respect to localization, which could account for the unaltered localization observed in the KO PP5 MEFs. Lack of a preference for PP5 explains the unaffected localization of PR-B in its absence. Taken together, our results show that although TPR specificity mostly controls receptor localization, altered equilibrium levels and absence of certain TPR proteins can potentially change the scenario.
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ABSTRACT

Steroid-receptor associated immunophilins not only act in a coordinated manner to establish and maintain receptor conformation optimal for signaling but also play a critical role in determining receptor localization. To demonstrate TPR preference in steroid receptor localization, we chose a mouse fibroblast cell line, which expresses high levels of endogenous GR, and a Chinese hamster ovary, derived cell line, WCL2, that over-expresses mouse GR localized in the nucleus in the unliganded condition. Immunofluorescence and fractionation studies showed that although GR is predominantly nuclear in the WCL2 cells, it is not tightly bound to this compartment and is released into the cytosolic fraction upon cell lysis. Comparing the differences in the equilibrium levels of FKBP51 and FKBP52 between L929 and WCL2 not only revealed a very cell-type specific expression pattern but also hinted at the possibility of differential recruitment.

Analysis of heterocomplex composition identified FKBP52 as the major TPR in WCL2 cells while FKBP51 was preferentially recruited into GR complexes from L929 cells. Having demonstrated the TPR preferences of GR in both cell types, we over-expressed FLAG-tagged FKBP51 and FKBP52 to determine if receptor subcellular localization could be altered by changing the equilibrium levels of these proteins. Receptor heterocomplexes in WCL2 showed a greater cytoplasmic distribution resulting from the recruitment of FLAG-tagged FKBP51, while distribution of GR in L929 cells remained unaffected by the distribution of flag-tagged FKBP52.

To assess if the subcellular localization of other steroid receptors as well hinged on their preference towards specific TPR proteins, GFP fluorescence and co-
immunoprecipitation assays were performed on receptorless COS-1 cells expressing either GFP-tagged wild or chimeric receptor constructs of GR and PR-B. Preference of GR towards FKBP51 remained unaltered while PR-B complexes mostly recruited FKBP52. Similar studies performed on chimeric receptor constructs identified a dual role of the ligand-binding domain (LBD), which is involved in controlling TPR specificity of receptor as well as subcellular localization. Finally, by selectively deleting specific TPR proteins, we show that loss of FKBP51 had a minor impact on localization of both receptors; loss of FKBP52 had a dramatic impact on PR localization while absence of PP5 did not alter localization of either receptor sub-type. Taken together these results show that steroid receptor preference for specific TPR controls overall subcellular localization although this can be overcome by equilibrium levels of cell-type specific expression.