# Control of Glucocorticoid and Progesterone Receptor Subcellular Localization by the Ligand-Binding Domain Is Mediated by Distinct Interactions with Tetratricopeptide Repeat Proteins[†]

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ABSTRACT: 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 was found in the cytoplasm in a complex containing PP5 and FKBP51, while the GR of WCL2 cells was nuclear and contained PP5 and FKBP52. Cyp40 did not interact with the GR in either cell line. To test whether FKBP interaction determined localization, we overexpressed Flag-tagged FKBP51 in WCL2 cells and Flag-FKBP52 in L929 cells. In WCL2 cells, the GR exhibited a shift to greater cytoplasmic localization that correlated with recruitment of Flag-FKBP51. In contrast, Flag-FKBP52 was not recruited to the 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. The GR−GFP construct localized to the cytoplasm, while the PR−GFP construct was predominantly nuclear. Similar to L929 cells, the 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 both TPR specificity and localization. Lastly, we analyzed GR and PR localization in cells completely lacking TPR. PR in FKBP52 KO cells showed a complete shift to the cytoplasm, while GR in FKBP51 KO and PP5 KO cells showed a moderate shift to the nucleus, indicating that both TPRs contribute to GR localization. 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.

Steroidal control of physiology requires the activation of steroid receptors (SR),[1] which serve as regulators of differential gene expression (*1, 2*). Prior to hormone binding, all members of the SR family are known to enter into large heteromeric complexes containing the molecular chaperone HSP90 and the cochaperone p23 (*3*). However, a number of additional cochaperones have been identified that seem to variably interact with SR complexes (*4, 5*). 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 (*6*). Indeed, TPR proteins enter into SR complexes by directly binding to HSP90 at its C-terminal TPR acceptor site (*7*−*9*). Interestingly, most studies suggest that the TPR acceptor site of HSP90 can accommodate only one TPR protein at a time (*10*−*12*). This fact means that several distinct SR heterocomplexes are possible, even in the same cell, based on TPR protein composition. Thus, many reports of interactions of SR with FKBP51, FKBP52, Cyp40, and PP5 have been published, 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 SRs, yet a competing school of thought exists. It holds that receptors can be preferentially regulated by one TPR over another. In this study, we address these competing models by assessing the contribution of

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[1] Abbreviations: SR, steroid receptor; GR, glucocorticoid receptor; PR, progesterone receptor; HSP90, heat shock protein 90; FKBP52, FK506-binding protein 52; FKBP51, FK506-binding protein 51; PP5, protein phosphatase 5; Cyp40, cyclophilin 40; TPR, tetratricopeptide repeat; LBD, ligand-binding domain; NLS, nuclear localization signal; CLS, cellular localization signal.

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FKBP51, FKBP52, Cyp40, and PP5 to the cellular distribution function of two receptors, glucocorticoid receptor (GR) and progesterone receptor (PR).

Since the early studies of Jensen and Gorski (*13, 14*), a central issue has been the location within the cell of hormone-free SR complexes. Because investigations with the first SR antibodies were carried out with estrogen and progesterone receptors (*15, 16*), 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 of glucocorticoid, mineralocorticod, and androgen receptors showed localization of these receptors to the cytoplasm (*17*−*20*). 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 GR, for example, is found in the cytoplasm of most cells, even in the very same cells where estrogen receptor or PR localizes to the nucleus (*21, 22*). More importantly, we now know that SRs control their own localization through functional regions residing within the hinge and ligand-binding domains (LBDs). Early work by Yamamoto and colleagues identified the presence of two nuclear localization signals, NLS1 and NLS2, on the GR (*23*). 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 (*24, 25*) 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 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 (*22*) 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 1−5 at the N-terminal end of the LBDs of both GR and PR (*22*). Interestingly, this region coincides with the putative signal transduction domain originally proposed by Pratt and colleagues (*26*), which serves as a principal site for the SR−HSP90 interaction (*27*). Because HSP90 is the mediator for TPR protein binding, and because FKBP52 is known to bind the motor protein dynein (*12, 28*), the involvement of the HSP90−FKBP52 complex in SR trafficking has been proposed, with most reports showing a role for FKBP52 in hormone-induced nuclear translocation of GR (*29*−*32*). However, the CLS−HSP90−TPR model data also suggest the intriguing possibility that differential incorporation of TPRs, such as FKBP51 and FKBP52, into receptor complexes could form the basis for known differences in localization among hormone-free receptors. 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 and, to a lesser extent, FKBP51. 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.

## EXPERIMENTAL PROCEDURES

*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 membrane was obtained from Millipore Corp. (Bedford, MA). Lifofectamine 2000 transfection reagent, OPTI-MEM, glycerol, and goat serum used for blocking in immunofluorescence were obtained from Invitrogen Corp. (Carlsbad, CA). FiGR monoclonal antibody against GR and rabbit polyclonal antibody against PP5 were generous gifts from J. Bodwell (Dartmouth Medical School, Hanover, NH) and M. 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 flourescein-conjugated goat anti-mouse IgG (401234) were 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 Eagle's medium (DMEM) containing 10% iron-supplemented newborn calf serum. The WCL2 cells (*47*) are derivatives of CHO cells subjected to rounds of methotrexate amplification after cotransfection with plasmids containing the cDNA for mouse wild-type GR and dihydrofolate reductase. WCL2 cells were maintained in 10 $\mu$M methotrexate. COS-1 cells (African green monkey kidney cell line, a generous gift from B. Rowan) were maintained using DMEM containing 10% iron-supplemented newborn bovine calf serum. Mouse embryonic fibroblasts (MEFs) 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 MEFs were generated by transfecting primary cells with vector for SV40-large T antigen.

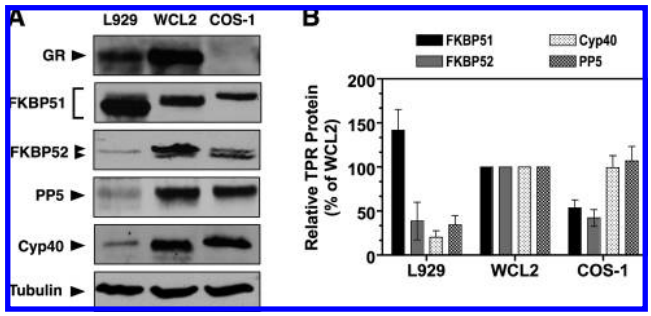


FIGURE 1: Western blot profile of TPR proteins in L929, WCL2, and COS-1 cells. (A) Expression levels of GR, FKBP52, FKBP51, Cyp40, and PP5 were measured by Western blotting of whole cell lysates from L929, WCL2, and COS-1 cells. Tubulin was blotted as a loading control. (B) Quantitation of TPR levels. Densitometric values were normalized as the percent of WCL2 and represent the means ± the standard error of the mean (sem) of three independent experiments.

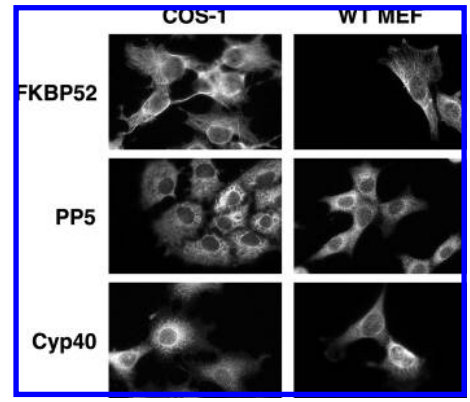


FIGURE 2: Comparison of the TPR intracellular distribution. Indirect immunofluorescence was performed in COS-1 and wild-type (WT) mouse embryonic fibroblast (MEF) cells using antibodies against FKBP52, PP5, and Cyp40. Results are representative of three independent experiments with at least 100 cells inspected per condition.

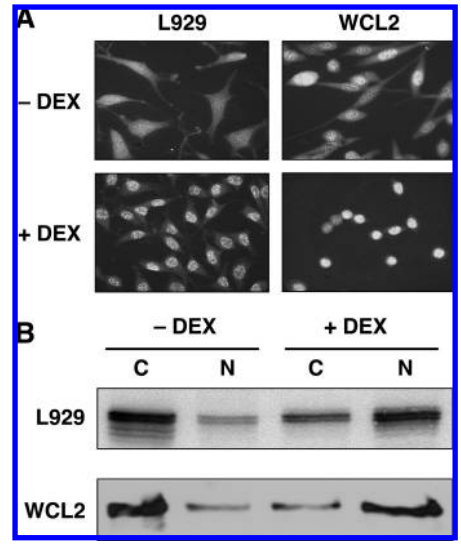


FIGURE 3: Comparison of hormone-free GR in L929 and WCL2 cells by fluorescence and fractionation. (A) GR localization in L929 and WCL2 cells was detected by indirect immunofluorescence using FiGR monoclonal antibody against receptor, as described in Experimental Procedures. Cells were treated with vehicle or dexamethasone (DEX, 1 $\mu$M for 1 h), as indicated. (B) L929 and WCL2 cells treated with or without dexamethasone (DEX, 1 $\mu$M, 1 h) were fractionated into cytosolic (C) and nuclear (N) extracts. Immunoadsorption was performed using the FiGR antibody against GR, followed by Western blotting. Results in panel B are representative of three independent experiments.

Transformed cells maintain normal MEF morphology with high proliferative activity.

*Transient Transfection.* For transient transfection, cells were plated on a 60 mm dish in DMEM containing 10% iron-supplemented calf serum prestripped 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 to the manufacturer's protocol. OPTI-MEM was removed after 5 h, and DMEM containing dextran-coated charcoal-stripped serum was added. All hormone treatments were conducted 16−20 h post-transfection for 1 h.

*Whole Cell Extraction.* Cells were washed and collected in 1× PBS followed by centrifugation at 1520*g* for 10 min. The supernatant was discarded, and the pellet was resuspended in 1× PBS. After a short spin at 20800*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 resuspended in 3 volumes of cold whole cell extract buffer [20 mM HEPES, 25% glycerol, 0.42 M NaCl, and 0.2 mM EDTA (pH 7.4)] with protease inhibitors and incubated on ice for 10 min. The samples were centrifuged at 100000*g* for 10 min at 4 °C. Protein levels were estimated spectrophotometrically. The supernatants were either stored at −80 °C or used immediately for Western 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, and 5% glycerol (pH 7.4)]. Lysates were centrifuged at 20000*g* for 30 min to generate cytosolic fractions (cytosol). All cytosols were used without freezing or storage. Immunoadsorption was performed by adding 10 $\mu$L of FiGR monoclonal IgG against GR or non-immune mouse IgG2A to aliquots of cytosol along with 50 $\mu$L 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, and 20 mM sodium molybdate (pH 7.4)] followed by elution of receptor heterocomplexes with 2× SDS sample buffer. For all immunoadsorptions performed in COS-1 cells after transient transfection of GFP-tagged receptors, 10 $\mu$L of anti-GFP mouse monoclonal IgG or control antibody was added to the cytosolic extract along with 50 $\mu$L of protein G-Sepharose. G-Sepharose instead of A-Sepharose was used to minimize nonspecific binding by the GFP moiety.

*Gel Electrophoresis and Western Blotting.* Samples were resolved on denaturing SDS gels (*48*) using a 7 to 14% acrylamide gradient 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 (Figures 3−5 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 M. Chinkers). Anti-Flag M2 monoclonal IgG [F-3165 (Sigma)] and anti-GFP mouse

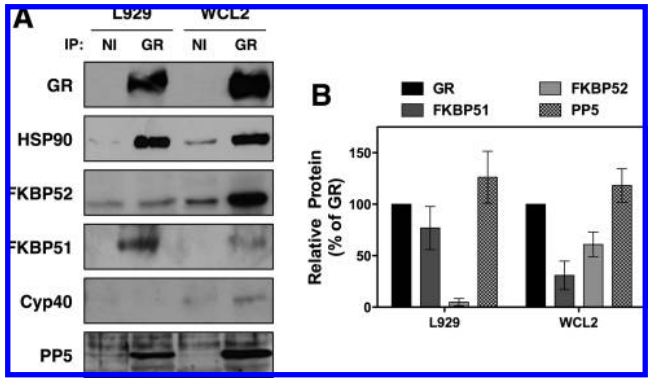


FIGURE 4: GR complexes in WCL2 cells recruit higher levels of FKBP52 and smaller amounts of FKBP51 compared to L929 cells. (A) Analysis of TPR content in GR heterocomplexes from L929 and WCL2 cells. Aliquots of L929 and WCL2 cytosol were immunoadsorbed with FiGR antibody against GR or nonimmune mouse 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 nonimmune values and normalization to the amount of GR protein under each condition. Values represent the means ± sem of three independent experiments. Cyp40 values are omitted because of a lack of interaction with either GR.

monoclonal IgG [sc-9996 (Santa Cruz Biotechnologies)] were used in the experiments depicted in Figures 5−7. After being probed, 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 a Bio-Rad GS-670 imaging densitometer.

*Indirect Immunofluorescence.* L929, WCL2, and COS-1 cells were seeded on laminin-coated coverslips in 60 mm dishes at densities of 50000−100000 cells/dish. Forty-eight hours later, cells were washed with HBSS (pH 7.4), fixed with ice-cold methanol for 10 min, and blocked for 30 min with 10% goat serum in PBS. Cells were then incubated with primary antibody for 1 h 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 three times with PBS, and the coverslips 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 coverslips in 60 mm dishes at densities of 300000−500000 cells/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

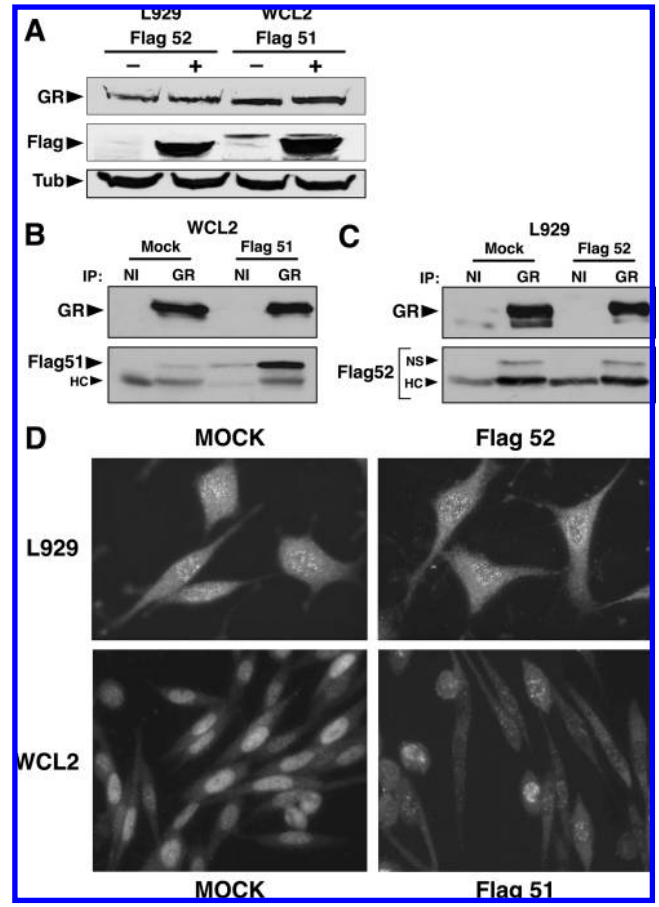


FIGURE 5: Overexpression of FKBP51 alters GR localization in WCL2 cells, with no effect of FKBP52 on L929 cell GR. (A) L929 and WCL2 cells were transiently transfected or not with Flag-tagged FKBP52 and Flag-tagged FKBP51, respectively. Whole cell extracts were analyzed for GR, Flag construct, and tubulin (loading control) by Western blotting. (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 nonimmune 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 described above, except Flag-tagged FKBP52 was used. Results in panels A−D are representative of three independent experiments.

at low laser power to avoid photobleaching. 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.

## RESULTS

As in most cells, 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 (*33*), 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 (*22*). 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 differential localization have yet to be discovered. Here, we exploit these differences to test the hypothesis that differential

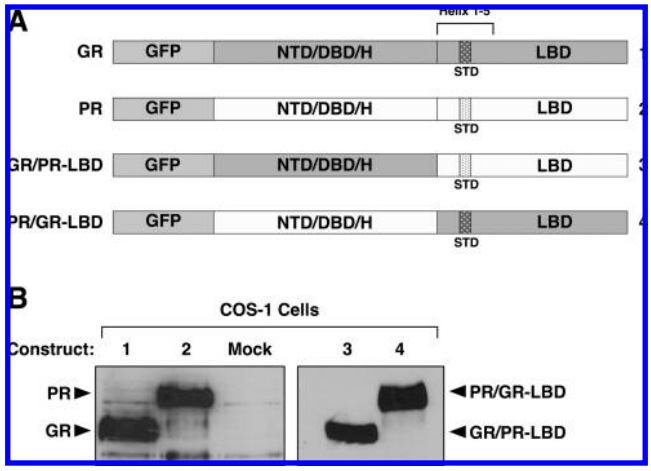


FIGURE 6: Domain structure of GFP-tagged wild-type and chimeric constructs of GR and PRB. (A) Schematic representation of wild-type human GR and human PRB linked to green fluorescent protein (GFP) at the N-terminal domain (NTD). Also shown are chimeric constructs in which the ligand-binding domains (LBDs) 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 (*22*). These helices coincide with the signal transduction domain (STD) previously shown by Pratt to be a major site for HSP90 interaction (*26, 27*). DBD, DNA-binding domain; H, hinge region. (B) Western blot profile confirming approximately equal expression of each construct (as numbered in panel A) 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.

recruitment of TPR proteins may form the basis for differential localization of hormone-free steroid receptor complexes. As a further test, we employed a series of mouse embryonic fibroblasts (MEFs) made from animals with targeted ablation of FKBP52, FKBP51, or PP5.

*Expression Profile and Subcellular Localization of TPR Proteins in L929, WCL2, and COS-1 Cells.* For reasons described above, we chose L929, WCL2, and COS-1 cells for the experiments depicted in Figures 3−7. Because interaction of the TPR protein with SRs could simply be a consequence of relative TPR protein expression levels, we measured the relative levels of FKBP51, FKBP52, PP5, and Cyp40 in each of these cell lines (Figure 1). At the outset, it should be noted that the quantitative Western blot procedure used throughout this work cannot measure true stoichiometry but can be used to determine ratios of the same protein across cell types and treatments. The results show that WCL2 cells have more FKBP52 than L929 and COS-1 cells, while levels of FKBP51 are the highest in L929 cells. Levels of Cyp40 and PP5 are comparable in WCL2 and COS-1 cells and are considerably lower in L929 cells.

We also considered the possibility that TPR localization, rather than concentration, could be the determining factor in SR distribution. We, therefore, performed indirect immunofluorescence for each TPR (except FKBP51) in L929, COS, WCL2, and wild-type (WT) and TPR-deficient MEF cells. MEF cells deficient in FKBP51, FKBP52, and PP5 were used to determine the specificity of our antibodies in the immunofluorescence assay. It should be noted that immunofluorescence data for FKBP51 are not shown, as we were not able to identify a suitable antibody using FKBP51 KO MEF cells as a control. Figure 2 shows the distribution of FKBP52, PP5, and Cyp40 in COS-1 and MEF cells. Similar results were obtained in L929 and WCL2 cells for each TPR (data not shown). FKBP52 exhibited a strong cytoplasmic signal with some nuclear content. In the cytoplasm, the FKBP52 signal exhibited a clear localization to the cytoskeletal network, a result consistent with earlier publications demonstrating colocalization of FKBP52 to microtubule filaments (*34, 35*) and the ability of FKBP52 to bind the motor protein dynein (*12*). The intracellular patterns of PP5 and Cyp40 were similar to each other, showing a strong perinuclear distribution. Thus, each TPR appears to have a well-defined distribution pattern that is also highly consistent across cell lines.

*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 (*33*). As seen in Figure 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 (Figure 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 (*30*). We consequently speculated that recruitment of FKBP52 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 (Figure 1), suggesting that recruitment of FKBP52 in place of FKBP51, due to naturally occurring FKBP52 overexpression, could lead to altered localization of GR. To test this hypothesis, we compared the FKBP composition of unliganded GR heterocomplexes in L929 and WCL2 cells (Figure 4). Because other TPRs could be involved in localization, we also determined the status of PP5 and Cyp40 in the GR complexes. Interestingly, the amount of PP5 recruited by GR was approximately 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 FKBP results were compared. As expected, GR in L929 cells contained FKBP51 with little or no FKBP52, but this ratio was reversed for the WCL2 GR.

*Overexpression of FKBP51 but Not FKBP52 Alters GR Localization.* To this point, our results suggest a model in which the equilibrium ratio of FKBPs is a major determinant of GR interaction and localization. To test this model, we performed a series of overexpression experiments using Flag-tagged FKBP51 and FKBP52. Figure 5A shows that comparable overexpression of tagged FKBP51 and FKBP52 was achieved in L929 or WCL2 cells without a change in GR expression levels. Co-immunoprecipitation analysis showed recruitment of newly expressed FKBP51 to the WCL2 GR complex (Figure 5B). Consistent with the model, GR

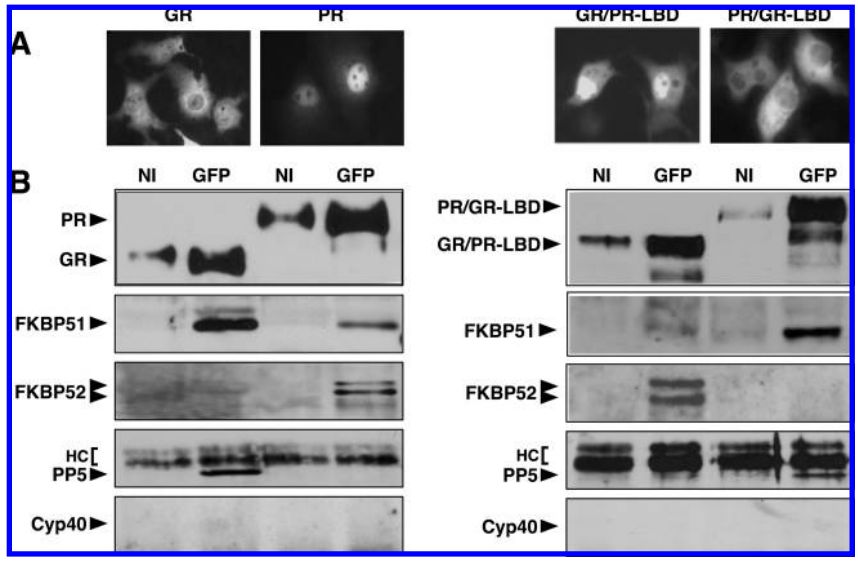


FIGURE 7: Ligand-binding domains of GR and PRB control differential localization and TPR specificity. (A) LBD controls GR and PRB localization. COS-1 cells were transfected with the indicated wild-type or chimeric receptor expression vectors. Fluorescence microscopy was performed on live, unfixed cells. (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 nonimmune 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. HC, antibody heavy chain. Results in panels A and B are representative of three independent experiments. In panel A, a minimum cohort of 100 cells per condition was inspected.

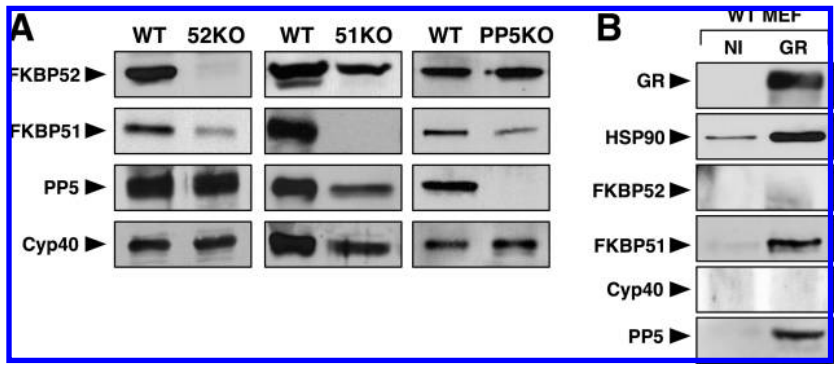


FIGURE 8: Characterization of MEF cells deficient in FKBP52, FKBP51, or PP5. (A) Western blot analysis of TPR proteins in MEF cells from TPR KO mice (see Experimental Procedures). (B) GR of WT MEF cells enters into complexes with FKBP51 and PP5. Cytosols from WT MEF cells were immunoadsorbed with FiGR antibody against GR or with nonimmune IgG (NI). Samples were split and analyzed by Western blotting for GR, HSP90, FKBP52, FKBP51, Cyp40, and PP5. Results in panels A and B are representative of five and two independent experiments, respectively.

localization was also changed upon FKBP51 overexpression, as GR was no longer confined to the nuclear compartment but showed at least a partial shift to the cytoplasm (Figure 5D). We next overexpressed Flag-tagged FKBP52 in L929 cells. Interestingly, recruitment of Flag-FKBP52 to the GR could not be detected using antibodies to the Flag epitope (Figure 5C) or FKBP52 itself (data not shown), even though levels of Flag-FKBP52 expression in the L929 cells were comparable to that achieved for Flag-FKBP51 in the WCL2 cells (Figure 5A). Because Flag-FKBP52 failed to interact with GR, it was not surprising that GR localization was unchanged following transfection (Figure 5D). On the basis of these results, it appears that the equilibrium model cannot be the only process controlling the interaction of FKBP 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 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 (*17, 36*), and compare its TPR specificity and localization to GR. To achieve this, wild-type (WT) and chimeric forms GR and PRB were obtained (a generous gift of S. Nordeen) which have green fluorescent protein (GFP) fused to the N-terminal domain (NTD) of each receptor (Figure 6A). The chimeras of GR and PRB were constructed so exchange of ligand-binding domains (LBDs) could be achieved. 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 chimera is constructed in an analogous manner. Expression vectors encoding the GR, PR, GR/PR−LBD, and PR/GR−LBD proteins were transiently transfected into receptor-less COS-1 cells so comparable levels of expression could be achieved (Figure 6B).

*TPR Specificity Correlates with Localization and Maps to the Ligand-Binding Domain.* Using the GR and PR constructs described above, Wan et al. showed that dif-

ferential intracellular localization of receptors was controlled by the ligand-binding domain (*22*). To determine the role played by TPRs in this process, we first confirmed the localization properties of these receptors (Figure 7A). In COS-1 cells, unliganded GR was predominantly cytoplasmic. In contrast, PRB was mostly nuclear in the absence of progestin agonist (R5020), although a small portion was observed in the cytoplasm, as previously reported (*36, 37*). 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 (Figure 7B). Like GR in L929 cells, wild-type GR in COS-1 cells was found to associate with FKBP51 and PP5, but not 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 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 the PR/GR−LBD chimera 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 and PR for FKBP52 but also for the first time show that the ligand-binding domain of each receptor is the site of this specificity.

*Analysis in Wild-Type and TPR-Deficient Mouse Embryonic Fibroblast Cells.* On the basis of 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, FKBP51 KO, and PP5 KO animals have been described elsewhere (*38, 39, 49*). As seen in Figure 8A, each KO MEF cell line is completely devoid of its respective TPR. The cell lines also had normal levels of expression for all TPR proteins, except for FKBP51 levels in FKBP52 and PP5 KO cells which were reduced by 51.8 and 59.6%, respectively. Thus, loss of TPR protein by targeted ablation does not result in compensatory overexpression of other TPRs. The mechanism behind the reduced level of FKBP51 expression in FKBP52 and PP5 KO cells is under investigation. We also performed co-immunoprecipitation analysis of GR complexes expressed in WT MEF cells (Figure 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 the absence of FKBP51 might affect localization of receptors, GFP−GR and GFP−PRB chimeras 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 (Figure 9). As expected, the GFP−GR chimera in WT MEF cells was found exclusively in the cytoplasm. In FKBP51 KO MEFs, however, a fraction of unliganded GR shifted to the nucleus. Altered localization was also observed for the GFP−PR chimera. 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 magnitude of the cytoplasmic PR signal was reduced, suggesting that FKBP51 determines the cytoplasmic localization of a subpopulation of PR.

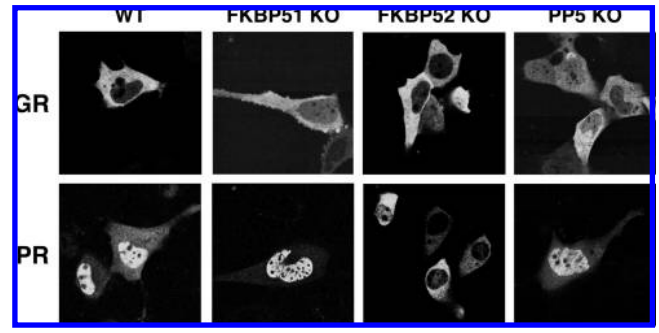


FIGURE 9: Analysis of GFP−GR and GFP−PRB chimeras by confocal microscopy in WT, FKBP51 KO, FKBP52 KO, and PP5 KO MEF Cells. MEF cells were transfected with the GFP−GR or GFP−PRB chimera, followed by a 1 h treatment with vehicle (shown) and confocal microscopy on live, unfixed cells. Cells were also treated with 100 nM dexamethasone or 20 nM R5020, as appropriate, for 1 h (data not shown; see the text for a discussion). In all cases, hormone treatment of receptor in TPR-deficient cells caused nuclear translocation equivalent to that seen in WT cells. A minimum cohort of 100 cells per condition was inspected for each condition.

*FKBP52 Deficiency Affects Only PRB Localization.* Figure 9 shows the results of expression of GFP−GR and GFP−PRB chimeras in FKBP52 KO MEF cells. The results suggest that FKBP52 does not play a crucial role in the localization of GR, as WT and FKBP52 KO MEFs had similar cytoplasmic distribution patterns for GR. These results were not surprising since the GR complex of MEF cells, like those of L929 and COS cells, does not interact with FKBP52 (Figure 8). Because FKBP52 KO cells express less FKBP51 than WT (Figure 8), a partial redistribution of GR to the nucleus might have been expected. However, GR complexes from FKBP52 KO cells contain the same amount of FKBP51 as GR in WT cells (data not shown). 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.

*Altered Localization of GR but Not PRB in PP5 KO Cells.* 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 (*40*), 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 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 downregulation of PP5 (*41*). To address this controversy, GR and PRB localization was assessed in PP5 KO MEF cells (Figure 9). GR in PP5 KO cells showed a partial redistribution to the nucleus similar to that seen in FKBP51 KO cells. This suggests that PP5, like FKBP51, contributes but is not essential to the cytoplasmic localization of hormone-free GR. Although PP5 KO cells show reduced levels of FKBP51 (Figure 8), reduction of this protein is likely not a contributor to the altered GR localization seen because GR complexes from PP5 KO cells have normal levels of FKBP51 (data not shown). 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.

## 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. On the basis of our results, we are able to draw 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 localization of hormone-free receptors. 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 overexpression 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 is transduced into differential recruitment of TPR proteins. Because the TPR interaction occurs through HSP90, it is possible that some type of intermolecular cross-talk may be occurring, with HSP90 as the 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 (*30*) is evidence that supports this model. Interestingly, support for intermolecular 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 the level of recruitment of PP5 (*32*). Similar work by the Scammell laboratory has shown that an elevated level of incorporation of FKBP51, especially the variant found in squirrel monkeys, into GR complexes leads to reduced hormone binding affinity (*42, 43*). Thus, if TPRs can control SR hormone binding function, presumably through HSP90 mediation, then SR control of TPR specificity via their LBD domains not only is reasonable but also 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 was 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 overexpressed in the WCL2 cells, incorporation of FKBP51 into GR complexes and a partial redistribution to the cytoplasm were seen. However, we were not able incorporate overexpressed FKBP52 into the GR complex of L929 cells or 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 that impose stringent controls on the SR−TPR interaction may be at play. This may explain why certain cells, such as human lymphocytes, have GR complexes with a high FKBP52 content (*42*). In the future, 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 subpopulation 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 the strong nuclear localization of most PRB is controlled by its association with FKBP52. 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 relocalize 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 of this already exists, as use of FK506 to remove FKBP51 from the GR complexes of L929 cells causes PP5 to take its place (*32*). For these reasons, it was important to test GR localization in the PP5 KO cells. Like FKBP51, loss of PP5 had a partial effect on GR redistribution to the nucleus. Thus, both FKBP51 and PP5 appear to play contributory and, perhaps, compensatory roles with respect to GR localization. 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.

As mentioned earlier, we have published our first descriptions of altered physiology in FKBP52 KO and FKBP51 KO mice (*38, 39*). Although this work provides data that corroborate 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 (*39*). Unfortunately, this genotype is an early embryonic lethal, making further investigation difficult. In the future, 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. Consistent with these 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 (*38*). Reduced ovulation and mammary gland ductal development also occur in FKBP52 KO females. On the basis of the PRAKO/PRBKO studies of Conneely and colleagues (*44*−*46*), those data suggest that loss of FKBP52 has a moderate effect on PRB functionality (ovary and mammary) but a more dramatic effect on PRA (uterus). Indeed, comparison of uterine PRA and PRB showed a stronger interaction by 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 more important 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 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 for combating endocrine disorders.

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