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Functional Protein Interactions in Steroid Receptor-Chaperone Complexes

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1. Introduction

Heat shock protein 90 (Hsp90) is unique in that it chaperones a select group of client proteins and assists their folding in preparation for key regulatory roles in cellular signalling. Steroid receptors are among the most extensively studied Hsp90 chaperone substrates and belong to the large nuclear receptor superfamily of hormone-activated transcription factors that respond to hormonal cues through conformational changes induced by hormone binding within the ligand-binding domain (LBD). In an ATP-dependent assembly process, high affinity hormone binding is achieved through the direct interaction of the steroid receptor LBD with Hsp90 and specific Hsp90-associated chaperones. After synthesis, steroid receptors enter the Hsp90 chaperoning pathway by initial assembly with Hsp40, followed by incorporation of Hsp70 and Hip. The binding of Hop and Hsp90 then generates an intermediate receptor complex which is further modified by the release of Hsp70 and Hop, allowing a transition of the receptor to hormone-binding competency. Recruitment of p23 leads to formation of mature receptor complexes capable of binding hormone with high affinity and characterized by the additional presence of one of the immunophilin cochaperones, FKBP51, FKBP52, CyP40 and PP5. This dynamic assembly of receptors to a hormone-activatable state, together with a selective functionality of receptors associated with specific Hsp90-immunophilin complexes provides mechanisms through which Hsp90 and the immunophilin cochaperones may regulate hormone-induced signalling events. This may occur directly by enhancing hormone binding as has been observed for AR, GR and PR associated with Hsp90-FKBP52 complexes or indirectly by facilitating nuclear import of receptor as seen

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subsequent to the hormone-induced exchange of FKBP51 by FKBP52 in GR-Hsp90 complexes. For more in depth summaries related to the mechanism and functional consequences of steroid receptor assembly with the Hsp90 chaperone machine, readers are referred to recent reviews (Echeverria & Picard, 2010; Picard, 2006; Pratt & Toft, 2003; Ratajczak *et al.*, 2003; Riggs *et al.*, 2004; Smith & Toft, 2008).

It is understood that ligand binding induces conformational changes within the steroid receptor LBD, facilitating release of Hsp90 and its cochaperones and exposing elements required for homodimerization, nuclear translocation and DNA binding. The mechanisms through which Hsp90 chaperone machinery regulates the physiological response to steroid hormones mediated by steroid receptors remain unclear. In early work, multiple approaches that included deletion analyses, peptide competition studies and use of the *in vitro* receptor-Hsp90 heterocomplex assembly system present in rabbit reticulocyte lysate were aimed at defining the regions within steroid receptors and Hsp90 responsible for interaction (Pratt & Toft, 1997). These revealed that the GR LBD was essential for formation of apo-GR-Hsp90 heterocomplexes and defined a ~100-amino acid minimal segment (human GR residues 550-653) required for high-affinity Hsp90-binding. The region contains the so-called signature sequence (human GR residues 577-596) that is conserved among steroid receptors, and may contribute to the stability of receptor-Hsp90 interaction. Despite the identification of this core Hsp90 interaction domain, other results suggested a role for nearly all of the LBD in GR association with Hsp90. Similar studies with PR and ERα also concluded that several regions throughout the LBD participate in the assembly of receptor-Hsp90 complexes, although for ERα the much less stable association of the LBD with Hsp90 requires a short upstream sequence (human ERα residues 251-71), located at the C-terminal end of the DNA-binding domain, to confer increased stability. Since Hsp90 has not been shown to bind directly to this upstream sequence, it has been proposed that the region may alternatively serve as a contact site for Hsp90 cochaperones (e.g. FKBP52) (Pratt & Toft, 1997).

Studies by the Toft laboratory, with mutants of chicken Hsp90α translated *in vitro* in reticulocyte lysate, have shown that the PR-Hsp90 interaction can tolerate deletion of the first 380-residues within the 728-amino acid chicken Hsp90α sequence to produce a hormone-activatable receptor. By contrast, selected regions (amino acids 381-441 and 601-677) in the C-terminal half of chicken Hsp90α were shown to be particularly important for PR-Hsp90 binding, with their deletion also interfering with receptor hormone responsiveness (Sullivan & Toft, 1993). An alternate approach by Baulieu and coworkers, in which human GR was coexpressed in baculovirus-infected insect cells with wild type or mutant chicken Hsp90α containing selective internal deletions (ΔA: 221-290; ΔB: 530-581; ΔZ: 392-419), revealed a loss of GR-Hsp90 interaction upon deletion of region A within the N-terminal domain, whereas deletions of regions B and Z afforded aggregated receptor-Hsp90 complexes in which receptor was unable to bind hormone (Cadepond *et al.*, 1993). An extension of these studies by the same laboratory, to chicken ERα and human MR, also concluded that deletion of the A domain in chicken Hsp90α negates interaction with both receptors (Binart *et al.*, 1995). None of the deletions affected ERα hormone-binding capacity, but MR failed to bind aldosterone with removal of region B. Although these investigations led to conflicting conclusions in relation to the role of the Hsp90 N-terminal domain in receptor interaction, it is appreciated that the introduced modifications may have caused

structural perturbations leading to a disruption of Hsp90 functions elsewhere in the protein, possibly hampering valid interpretation of the results (Pratt & Toft, 1997).

Recent developments have led to the crystallographic analysis of steroid receptors, as well as Hsp90 and several of its cochaperones. At the same time, the use of the yeast two-hybrid system has revealed novel interactions between specific steroid receptors and selected cochaperones involved in the Hsp90 chaperoning pathway. Additionally, further insight is now available into the mechanism(s) that underlie the potentiation of AR, GR and PR by FKBP52. This review provides a summary of this recent progress with a focus on steroid receptor, Hsp90 and cochaperone contact domains that mediate interactions important for steroid receptor function.

2. Hsp90-steroid receptor interactions

2.1 GR LBD sub-regions required for assembly of apo-GR-Hsp90 complexes; GR structure

Further endeavours to identify sequences within the GR LBD critical for Hsp90 recognition were undertaken jointly by the Simons and Pratt laboratories. In initial studies using COS-7 cell-expressed receptor chimeras comprising glutathione S-transferase (GST) fused to the N-terminal end of an intact rat GR LBD and testing for recovered Hsp90, they found that a 7-residue amino-terminal truncation of the LBD eliminated both Hsp90 and steroid binding (Xu *et al.*, 1998). This allowed them to determine the 7-amino acid sequence, TPTLVSL (equivalent to amino acids 547-553 in rat GR and residues 529-535 in human GR, see Fig. 4), to be essential for the GR-Hsp90 interaction. Alignment of this sequence with the corresponding region in other steroid receptors revealed a conserved hydrophobic domain contained within helix 1 of the receptor LBD structure. It was proposed that the sequence defined a structure important for the unfolding of the hormone binding pocket, permitting steroid access and resulting in the exposure of a hydrophobic contact domain for stable Hsp90 interaction (Xu *et al.*, 1998). Extending the 7-amino acid sequence to include Leu554 in rat GR (Leu536 in human GR), gave the sequence TPTLVSLL and led to the recognition of the LXXLL protein-protein interaction motif within helix 1 (Giannoukos *et al.*, 1999). Such motifs have previously been shown to mediate interactions between transcriptional coactivators and members of the steroid/nuclear receptor super family (Ratajczak, 2001). Mutation of the first two leucine residues within the motif (L550S/L553S in rat GR) caused an increased rate of steroid dissociation, resulting in a dramatic loss of transcriptional activity. From a predicted GR structure, the GR LBD was seen as a “hinged pocket” with helices 1-6 comprising one side of the steroid-binding domain. In this model, the LXXLL motif within helix 1 was proposed to function as a hydrophobic clasp, helping to close one end of the steroid binding pocket by forming intramolecular contacts with residues in helices 8 and 9 on the opposite arm of the pocket, as well as residues in helix 3 and the intervening loop between helices 3 and 4 (Giannoukos *et al.*, 1999). The LXXLL motif was proposed then to play a key role in stabilizing GR LBD tertiary structure and would, as a consequence, make important contributions to steroid binding activity.

A mutational study of specific rat GR LBD residues within the previously defined minimal high affinity binding segment for Hsp90 revealed that alanine substitution of the conserved

Pro643 (analogous to human GR Pro625) profoundly reduced both the stability of the GR-Hsp90 heterocomplex, as well as transcriptional activity, despite retaining almost normal hormone-binding affinity (Caamano *et al.*, 1998). The negative effect on transcriptional function was related to a defect in nuclear translocation for the mutated receptor. Together the results strengthened the case for the requirement of Hsp90 as a critical component of steroid receptor signalling and identified an essential role for proline residue 643, located within an exposed hydrophobic loop between helices 5 and 6 in the receptor, in maintaining the apo-GR-Hsp90 interaction.

The x-ray structure of the human GR LBD, liganded to dexamethasone, resembles those for AR and PR, bound to their respective agonists and confirmed a helical sandwich arrangement for the steroid binding pocket (Bledsoe *et al.*, 2002). Pro625 was shown to be a key residue of a novel receptor dimerization interface involving reciprocal hydrophobic interactions between the helix 5-6 loop residues, Pro625 and Ile628 from each LBD and a hydrophobic bond network between the LBDs involving residues within the helix 1-3 loops (see Fig. 4). Since Pro625 is also central to the stability of GR-Hsp90 heterocomplexes, the finding suggested an overlap between the interface for receptor dimerization and an important contact domain for Hsp90. Indeed, this may form part of the mechanism that allows the Hsp90 chaperone complex to restrict transactivation of receptor in the absence of hormone (Picard, 2006). In comparison to GR, studies have revealed that ERα is less reliant on Hsp90 regulatory control over its hormone-dependent function (Picard *et al.*, 1990), allowing the ERα LBD to mediate dimerization in the absence of hormone *in vivo* (Aumais *et al.*, 1997). ERα homodimer formation in the LBD is mediated through helix 10, thus differing in configuration to that of GR (Bledsoe *et al.*, 2002). It is of interest that for ERα, substitution of a valine residue for Gly400, also within the helix 5-6 loop of the ERα LBD, induces a conformational change that destabilizes the receptor LBD, promoting a stronger, more stable association with Hsp90, similar to that for GR and rendering receptor transactivation more hormone-dependent (Aumais *et al.*, 1997).

2.2 Hsp90 structure; Amphipathic helices 1 and 2 in the Hsp90 C-terminal domain with potential for GR-binding

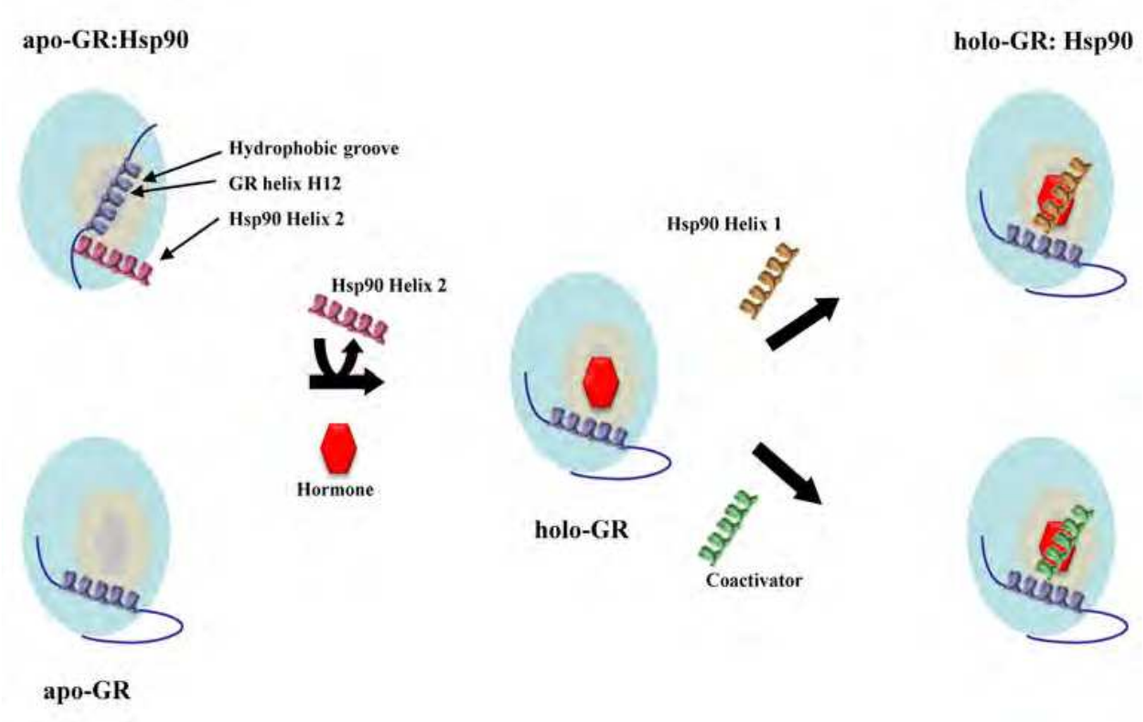
The x-ray crystal structure of the C-terminal dimerization domain of htpG, the *Escherichia coli* Hsp90, was recently solved by Agard and coworkers, revealing a dimerization motif defined by a four-helix bundle interface derived from the interaction of helices 4 and 5 of one monomer with equivalent helices from a second monomer (Harris *et al.*, 2004). The structure also identified helix 2, a flexible, solvent exposed amphipathic helix, as a potential chaperone substrate-binding site. Hydrophobic residues within helix 2 are strongly conserved in Hsp90 homologues across species, suggesting an important underlying function. This was supported by other studies in which deletion of a region encompassing the corresponding helix 2 sequence in yeast Hsp82 impaired viability (Louvion *et al.*, 1996), while the point mutation, A587T, which defines the start of the helix, compromised the ability of Hsp82 to promote GR activity and caused a general reduction in Hsp90 function (Nathan &Lindquist, 1995). Core hydrophobic residues within the helix 2 sequence were observed to share sequence similarity with helix 12 of steroid receptors, leading to a proposal that Hsp90 helix 2 acts as a receptor helix 12 mimic in apo-receptor-Hsp90

complexes, occupying the normal activation function 2 (AF2) position of helix 12 following hormone binding (Jackson *et al.*, 2004). Structural elucidation of full-length yeast Hsp90 (Ali *et al.*, 2006) allowed the recognition of helix 1, also consisting of a solvent-exposed, hydrophobic surface within the Hsp90 C-terminal domain, as a possible contact site for protein-protein interactions (Fang *et al.*, 2006). The highly conserved hydrophobic sequence of this helix closely matches the LXXLL recognition motif of the Steroid Receptor Coactivator/p160 family of coactivators that modulate receptor transcriptional activity by interacting with the AF2 agonist-induced hydrophobic groove of nuclear receptors (Ratajczak, 2001).

2.3 Flexible positioning of receptor LBD helix 12; Hsp90 helix 2 induces apo-GR helix 12 to adopt the GR-RU486 antagonist conformation

Recent studies by Darimont and coworkers have confirmed that Hsp90 helix 2 stabilizes unliganded GR by engaging apo-GR at the position normally occupied by receptor helix 12 in response to hormonal activation and forcing the flexible helix 12 to bind to the hydrophobic groove, at the same time preventing receptor interaction with coactivators (Fang *et al.*, 2006). The resulting structure corresponds to the native conformation of unliganded GR, with an orientation of helix 12 similar to that in antagonist (RU486)-bound GR (Fang *et al.*, 2006; Kauppi *et al.*, 2003). On agonist binding, hormone-induced conformational changes within the LBD of holo-GR promote the replacement of Hsp90 helix 2 by receptor helix 12, causing loss of Hsp90 chaperone machinery and establishing the AF2 contact domain for coactivator interaction. Alternatively, the new structure might facilitate Hsp90 helix 1 binding to the hydrophobic groove. Since Hsp90 helices 1 and 2 are proximally located at the Hsp90 C-terminus, this exchange of receptor-Hsp90 interactions, which is partly determined by the dynamics of receptor helix 12, may likely be achieved within the one receptor-Hsp90 complex (Fig. 1).

The hormone-induced progression from apo- to holo-GR-Hsp90 complexes, through changes in the mode of receptor-Hsp90 interaction resulting from altered receptor LBD conformation, provides a suitable model for visualising the transition between inactive and active receptor that may also involve the participation of Hsp90 cochaperones such as FKBP51 and FKBP52. Although FKBP51 is the preferred cochaperone in mature GR-Hsp90 complexes (Barent *et al.*, 1998; Nair *et al.*, 1997), FKBP52 has been shown to promote increased GR hormone binding affinity and to potentiate the transcriptional activity of the receptor (Riggs *et al.*, 2003). It is possible that the observed hormone-induced interchange of FKBP51 by FKBP52 in GR-Hsp90 complexes, resulting in the favoured nuclear translocation of receptor complexes (Davies *et al.*, 2002), might be initiated by a change in GR LBD conformation elicited by the transfer of receptor interaction from Hsp90 helix 2 to helix 1, both helices being close to the common TPR binding site for immunophilin cochaperones in the C-terminal region of Hsp90. Unique steroid receptor LBD conformations then might be an important determinant of receptor preferences for specific immunophilin cochaperones within receptor-Hsp90 complexes (e.g. FKBP51 in GR, PR and MR complexes (Barent *et al.*, 1998; Nair *et al.*, 1997); PP5, the major cochaperone in GR complexes (Silverstein *et al.*, 1997) and CyP40, the prevalent immunophilin in ER complexes (Ratajczak *et al.*, 1990)), allowing these cochaperones to potentially modulate receptor function (Ratajczak *et al.*, 2003; Smith & Toft, 2008).



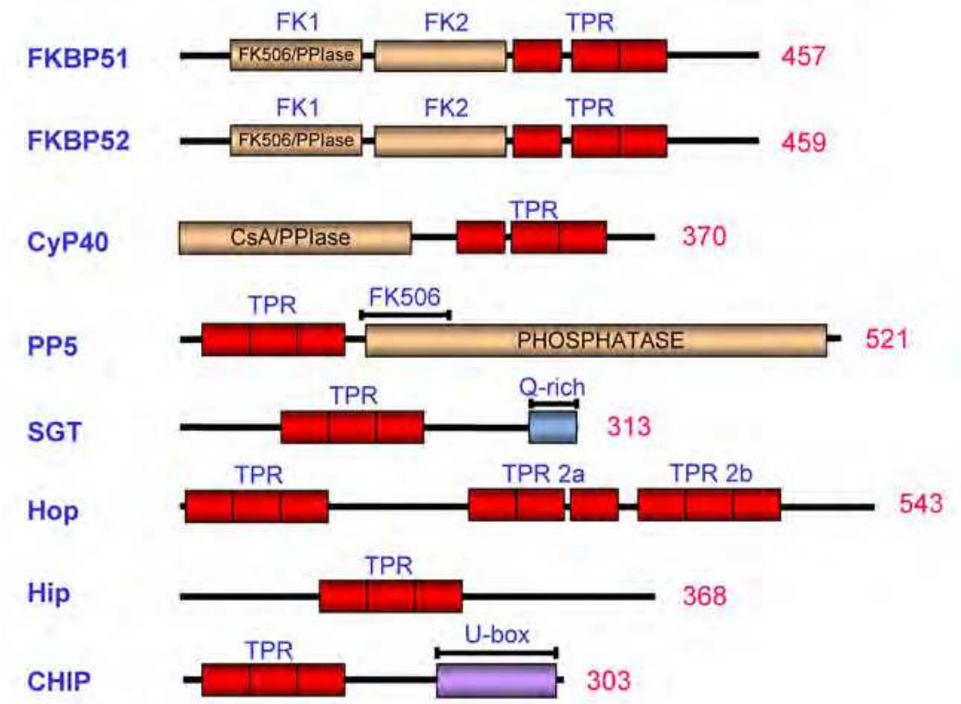
Hsp90 helix 2 binds apo-GR at the position normally occupied by GR helix H12, forcing H12 to dock within the hydrophobic groove, thus stabilizing the unliganded hormone-binding pocket. With hormone binding, GR H12 replaces Hsp90 helix 2 providing contacts for AF2-interacting coactivators or for Hsp90 helix 1.

Fig. 1. Hsp90 interactions with apo-GR and holo-GR.

3. Hsp90/Hsp70-cochaperone interactions

3.1 TPR cochaperones

Folding of newly synthesized peptides to functionally mature proteins, such as steroid receptors, is actively regulated by Hsp70 and Hsp90 with their cochaperones in what is known as the Hsp70/Hsp90-based chaperone machinery (Pratt & Toft, 2003). Cochaperones can regulate the nucleotide status, and thus function, of Hsp70 and Hsp90, and deliver non-native proteins to their respective polypeptide-binding domains for folding. Those cochaperones that regulate Hsp70 include Hsp40, Hsc70-interacting protein (Hip), Hsp-organizing protein (Hop) and small glutamine-rich TPR protein (SGT), while Hsp90 is regulated by cochaperones that include Hop, p23, PP5, CyP40, FKBP51 and FKBP52. C-terminal of Hsp70-interacting protein (CHIP) is another cochaperone that regulates both Hsp70 and Hsp90. Fig. 2 shows the domain architecture of the immunophilin and other TPR cochaperones with an established role in Hsp70 and/or Hsp90 chaperone function.



TPR domains are depicted in red whilst other specialized functional domains are highlighted in other various colours and labelled accordingly. Abbreviations: FKBP, FK506-binding protein; PPIase, peptidylprolyl isomerase; TPR, tetratricopeptide repeat; CyP40, cyclophilin 40; CsA, cyclosporin A; PP5, protein phosphatase 5; SGT, small glutamine-rich TPR protein; Hop, Hsp-organizing protein; Hip, Hsc70-interacting protein; CHIP, C-terminal of Hsp70-interacting protein.

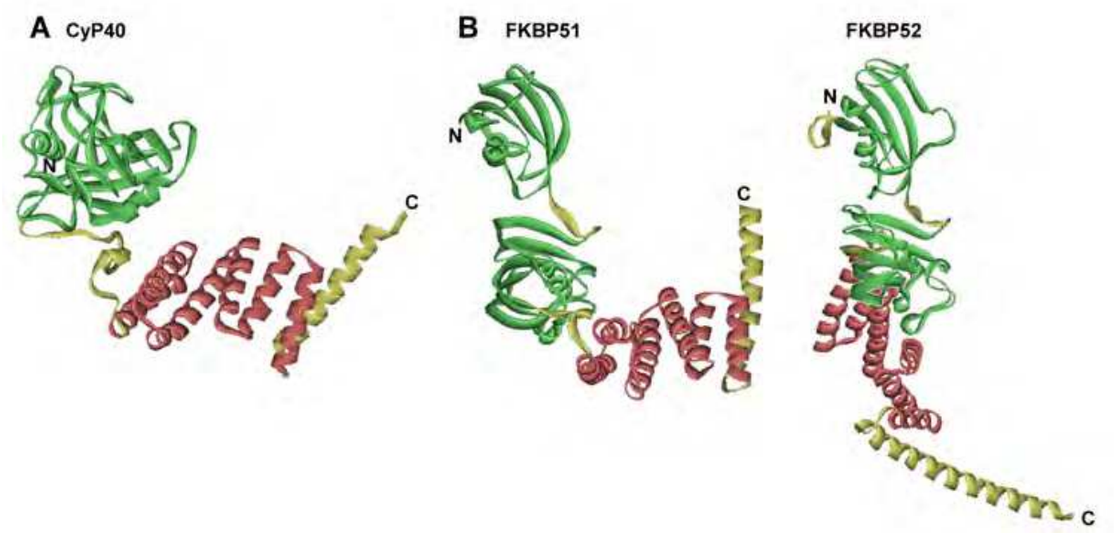
Fig. 2. Schematic presentation of the domain structures of TPR-containing proteins associated with the Hsp70/Hsp90 chaperone machinery.

Since the crystallization of the PP5 TPR domain, the structures of several other steroid receptor-associated TPR-containing proteins have been solved. There are now full-length structures available for bovine CyP40, human FKBP52, PP5 and Hop, human and squirrel monkey FKBP51, and mouse CHIP, as well as the structure of the human SGT TPR domain. It is known that TPR domains in these proteins can mediate interactions with Hsp70 and/or Hsp90 (Angeletti *et al.*, 2002; Smith, 2004), but in addition to their Hsp-recognition domains, each also possesses other localized functional domains important for their own conformation and/or the regulation of associated proteins.

3.1.1 CyP40, FKBP51 and FKBP52

CyP40 and the two FKBPs have a similar structural arrangement, each possessing an N-terminal binding site for the immunosuppressants cyclosporin A or FK506, respectively, and a C-terminal TPR domain (Sinars *et al.*, 2003; Taylor *et al.*, 2001; Wu *et al.*, 2004). The cyclophilin domain of CyP40 is similar to other single-domain cyclophilins (Kallen *et al.*, 1998). In FKBP51 and FKBP52, FK506 binds to the first of two FKBP domains, termed FK1, while the second domain, called FK2, lacks drug-binding activity. Bound immunosuppressants inhibit the peptidylprolyl isomerase (PPIase) activity of the

cyclophilin and FK1 domains, which may be important for target protein regulation by direct or indirect association. Fig. 3 provides a structural comparison between CyP40, FKBP51 and FKBP52 immunophilin cochaperones.



A, CyP40 and **B,** FKBP51, FKBP52. The CsA-binding domain (CyP40) and FK regions (FKBP51 and FKBP52) are shown in green. Core TPR domains for CyP40, FKBP51 and FKBP52 are depicted in red, with the final extended helices, at the C-terminal ends of each protein, shown in yellow.

Fig. 3. Ribbon representations of molecular structures of TPR-containing proteins.

3.1.2 PP5

PP5 is a phosphatase that dephosphorylates serine and threonine residues on target proteins (Barford, 1996; Cohen, 1997). Crystallisation of the full-length phosphatase in the absence of ligands or binding partners revealed the structural organization of the autoinhibited form of PP5 (Yang *et al.*, 2005). The TPR domain in PP5 is oriented to the N-terminus and is linked to a C-terminal phosphatase catalytic domain followed by a short C-terminal subdomain. In this inactive conformation, the TPR domain engages with the catalytic domain in such a way as to restrict target protein access to the enzymatic site, and this structure is stabilized by the C-terminal subdomain. Suppression of catalytic activity can be abolished by an allosteric conformational change that disrupts the TPR-catalytic domain interface, and this can be induced upon binding of polyunsaturated fatty acids or Hsp90 to the TPR domain (Chen & Cohen, 1997; Ramsey & Chinkers, 2002; Skinner *et al.*, 1997).

3.1.3 Hop

Hop plays a dual role in mature steroid receptor complex assembly by recruiting Hsp90 to preformed Hsp70-receptor complexes and inhibiting the ATPase of Hsp90 for client loading onto the chaperone for subsequent folding (Chen *et al.*, 1996b; Chen & Smith, 1998; Dittmar *et al.*, 1996; Kosano *et al.*, 1998; Prodromou *et al.*, 1999; Siligardi *et al.*, 2004). Hop has an N-terminal TPR domain (TPR1) followed by an aspartic acid/proline (DP)-rich region, and two more adjacent TPR domains (TPR2a and TPR2b) followed by a second DP-rich region.

3.1.4 Hip

Hip functions as a transient component of native steroid receptor complexes and enters the assembly cycle once Hsp70 ATPase activity has been stimulated by Hsp40 (Frydman & Höhfeld, 1997; Höhfeld *et al.*, 1995). Hip acts to stabilize the ADP-bound state of Hsp70 that is necessary for high affinity interaction with unfolded substrates (Frydman &Höhfeld, 1997; Höhfeld *et al.*, 1995). Structurally, Hip consists of an N-terminal oligomerization domain that is important for the functional maturation of GR in yeast (Nelson *et al.*, 2004), a central TPR domain and an adjacent highly charged region which are both required for Hsp70 binding (Prapapanich *et al.*, 1996b) and a C-terminal DP-rich domain that helps direct the intermediate stage recruitment of Hop-Hsp90 during assembly of steroid receptor complexes (Prapapanich *et al.*, 1998).

3.1.5 CHIP

The cochaperones described above are involved in maintaining an activatable conformation of Hsp70/Hsp90-dependent "clients", but TPR proteins also function to mediate the degradation of misfolded proteins, indicating a role in quality control (Cyr *et al.*, 2002). Selection of proteins for degradation is mediated by E3 ubiquitin ligases, and CHIP is a member of this enzymatic class (Jiang *et al.*, 2001; Murata *et al.*, 2001). CHIP has an N-terminal TPR domain and a C-terminal U-box domain that mediates its ligase activity, which promotes ubiquitylation of target substrates prior to their degradation by the proteasome.

3.1.6 SGT

Human SGT binds to viral protein U (Vpu) and Group specific Antigen, 2 proteins associated with human immunodeficiency virus-1, and the rat homologue was identified as an interactor of the non-structural protein NS-1 of the parvovirus H-1. The central TPR domain in SGT is flanked by an N-terminal dimerization domain and a C-terminal glutamine-rich domain involved in association with type 1 glucose transporter (Callahan *et al.*, 1998; Cziepluch *et al.*, 1998; Liou & Wang, 2005).

3.2 Regulation of Hsp70 and Hsp90 ATPases by TPR cochaperones

Both Hsp70 and Hsp90 require ATP for their functional association with substrates (Pratt & Toft, 2003). In the case of a steroid receptor, Hip binding to the N-terminal ATPase domain of Hsp70, possibly through a unique TPR binding site located within this region (see below), stabilizes the Hsp70-receptor complex (Frydman & Höhfeld, 1997; Höhfeld *et al.*, 1995) in a step that may be important for recognition by Hop and loading of the receptor onto Hsp90 for further processing. Hop contains three distinct TPR domains (TPR1, TPR2a, TPR2b) (Fig. 2), with TPR1 and TPR2a providing anchor points for the C-terminal EEVD peptides of Hsp70 and Hsp90, respectively. These specific interactions, coupled with domain-domain interactions, also involving its TPR domains, allow Hop to play a key role in coordinating the actions of Hsp70 and Hsp90 (Carrigan *et al.*, 2006; Chen *et al.*, 1996b; Chen & Smith, 1998; Odunuga *et al.*, 2003; Prodromou *et al.*, 1999; Ramsey *et al.*, 2009; Scheufler *et al.*, 2000). While the TPR acceptor site for Hop in the C-terminal region of Hsp90 serves to anchor the

cochaperone, studies have shown that Sti1, the yeast homologue of Hop, markedly inhibits the ATPase activity of yeast Hsp90 through secondary interactions that block the ATP-binding pocket in the Hsp90 N-terminal domain (Prodromou *et al.*, 1999). By directly competing with Sti1 for binding to Hsp90, the CyP40 yeast homologue Cpr6 can negate the Sti1-mediated blockade of Hsp90 ATPase activity following TPR protein exchange (Prodromou *et al.*, 1999). In contrast, in vitro studies with human Hop determined that the cochaperone had no influence on the weak basal ATPase activity of human Hsp90, but significantly inhibited the increased rate of ATP hydrolysis by Hsp90 in response to interaction with the ligand binding domain of GR, an established Hsp90 client protein (McLaughlin *et al.*, 2002). On the other hand, FKBP52, which like CyP40 binds competitively with Hop to the C-terminal TPR interaction site of Hsp90, was shown to enhance Hsp90 ATPase activity stimulated by GR (McLaughlin *et al.*, 2002). This control over ATP utilization is important for the functional activity of newly synthesized substrates, but ATPase regulation is also required for the degradation of improperly folded substrates. CHIP can bind Hsp70 and inhibit Hsp40-stimulated Hsp70 ATPase activity, and has been reported to deplete cellular GR levels (Ballinger *et al.*, 1999; Connell *et al.*, 2001). Therefore, CHIP can be regarded as a degradatory cochaperone of Hsp70 and Hsp90. SGT negatively regulates Hsp70 such that the chaperone has a reduced ability to refold denatured luciferase (Angeletti *et al.*, 2002).

3.3 Determinants of Hsp70 and Hsp90 interaction with TPR cochaperones

Deletion studies were the first to demonstrate that TPR domains mediated binding to Hsp90 (Barent *et al.*, 1998; Chen *et al.*, 1996a; Radanyi *et al.*, 1994; Ratajczak & Carrello, 1996). Determination of the TPR domain structure of PP5 revealed that the packing of adjacent TPR units generated an exposed groove capable of accepting a target protein peptide (Das *et al.*, 1998). Although TPR motifs are highly degenerate, they display a consistent pattern of key residues important for structural integrity. The two α-helical sub-domains in each TPR motif are arranged such that the groove is mainly composed of residues from the A helix of each repeat, while B helix residues are buried to form the structural backbone of the superhelix, and this groove forms a critical Hsp recognition surface.

In a PP5 mutagenesis study, Russell and coworkers carefully selected A helix residues with side-chains extended into the groove and identified four basic residues important for PP5-Hsp90 interaction (Russell *et al.*, 1999). These amino acids are highly conserved in other Hsp90-binding TPR proteins, and mutation of aligned residues in CyP40 confirmed their importance in Hsp90 recognition (Ward *et al.*, 2002). The key recognition sequence for the TPR domain in these proteins is the EEVD peptide located at the extreme C-terminus of Hsp90 (Carrello *et al.*, 1999; Chen *et al.*, 1998; Young *et al.*, 1998), which is conserved in Hsp70. Crystallization of individual Hop TPR domains with Hsp70 and Hsp90 N-terminally extended EEVD peptides has defined the mechanism of TPR domain-peptide interaction (Scheufler *et al.*, 2000). The TPR1 domain of Hop binds to Hsp70, while the TPR2a domain mediates Hsp90 recognition (Chen *et al.*, 1996b; Lassle *et al.*, 1997). The groove in each TPR domain accommodates their respective peptide in an extended conformation where the ultimate aspartate residue is tightly held by electrostatic interactions with TPR residue side-chains in a two-carboxylate clamp. Additional EEVD contacts involve hydrogen-bonding, while amino acids upstream of the EEVD enhance the affinity of the peptides for TPR

domains and mediate specificity of Hsp70 and Hsp90 to TPR1 and TPR2a, respectively. Notably, Hop TPR2a provides an example of where an additional sequence within the TPR domain doesn't disrupt the overall structure. TPR2a contains an insertion between units 2 and 3 that extends the helices by a single turn but does not impact Hsp90 peptide recognition (Scheufler *et al.*, 2000).

The Hsp90 dimerization domain, located in the C-terminal region upstream of the MEEVD peptide, contributes to TPR cochaperone recognition (Chen *et al.*, 1998) and contains the putative binding site for novobiocin, a coumarin-based Hsp90 inhibitor (Marcu *et al.*, 2000). *In vitro* studies demonstrated that novobiocin had a differential effect on Hsp90-immunophilin cochaperone interaction, suggesting that the TPR cochaperones modulate Hsp90 function through distinct contacts within the Hsp90 C-terminal domain (Allan *et al.*, 2006).

Although EEVD interactions with the TPR domain groove are critical for Hsp binding, regions outside of the TPR domains are also important in mediating recognition. TPR domains are typically followed by a seventh α-helix that packs against and extends beyond the TPR domain and has been shown to be involved in binding Hsp90 in addition to the TPR domain. FKBP51 and FKBP52 have different affinities for Hsp90 and are assembled differentially with specific receptor complexes, and these differences map in part to sequences C-terminal of their respective TPR domains (Barent *et al.*, 1998; Cheung-Flynn *et al.*, 2003; Pirkl & Buchner, 2001). The charge-Y motif was identified and found to be essential for FKBP-Hsp90 interaction, which was also confirmed for CyP40, but sequences further downstream in FKBP51 and FKBP52 differentially regulated Hsp90 binding (Allan *et al.*, 2006; Cheung-Flynn *et al.*, 2003; Ratajczak & Carrello, 1996). The acidic linker flanking the N-terminus of the CyP40 TPR domain was also shown to be important for efficient interaction (Mok *et al.*, 2006; Ratajczak & Carrello, 1996). Although an interaction partner for Hop TPR2b has yet to be identified, mutations in TPR2b reduced Hop interaction with both Hsp70 and Hsp90, while mutations in the C-terminal DP-rich region inhibited Hop binding to Hsp70 (Chen & Smith, 1998; Nelson *et al.*, 2003).

3.4 Alternative modes of Hsp70 and Hsp90 recognition by TPR cochaperones

Like Hop, CHIP binds to both Hsp70 and Hsp90 (Ballinger *et al.*, 1999; Connell *et al.*, 2001), but CHIP interacts with either of these major chaperones through a single TPR domain. Recent elucidation of the binding of Hsp90 C-terminal peptide (NH_2-DDTSRMEEVD) with the CHIP TPR domain has revealed that the peptide sequence is not accommodated in an extended conformation as for Hop, but turns at the methionine residue and becomes buried within a hydrophobic pocket (Zhang *et al.*, 2005). This pocket can accommodate either the methionine or isoleucine that lies immediately upstream of the EEVD sequence in Hsp90 and Hsp70, respectively, and the peptide is twisted, negating the role of upstream residues in conferring the same specificity seen in binding Hop TPR domains. SGT also recognizes Hsp70 and Hsp90 via its single TPR domain, but possibly through a different mechanism to that described for CHIP as SGT lacks the residues that form the hydrophobic pocket which allows the respective C-terminal peptides in the chaperones to twist (Dutta & Tan, 2008).

Hydrophobic pockets themselves may also be important structural features within TPR domains that confer Hsp specificity, as the crystal structure of Hop TPR2a with the non-cognate Hsp70 peptide shows the hydrophobic pocket to be less accommodating for the Ile

(-5) residue in the extended Hsp70 peptide than Met (-5) in the extended Hsp90 peptide, with the notable feature of a lack of bending by the Hsp70 peptide, such as with CHIP, to perhaps enhance affinity for TPR2A (Kajander *et al.*, 2009).

General cell UNC-45 (GCUNC-45), a member of the UNC-45/Cro1/She4p (UCS) protein family, is a TPR protein that regulates PR chaperoning by Hsp90 by preventing activation of Hsp90 ATPase activity (Chadli *et al.*, 2006). Hsp90-binding experiments in the presence of Hop revealed a novel GCUNC-45 TPR recognition site in the N-terminal domain of Hsp90, which also bound FKBP52 (Chadli *et al.*, 2008a). Further analysis defined a non-contiguous EEVD-like motif, centered in and around the Hsp90 N-terminal ATP-binding pocket, arranged in a structural conformation that can recognize TPR domains. Nucleotide binding negatively regulates the interaction. These authors also alluded to CyP40 binding to the N-terminal interaction motif, although Onuoha and coworkers have recently confirmed CyP40 interaction only with the C-terminal domain of Hsp90 (Onuoha *et al.*, 2008). GCUNC-45 is the first cochaperone to display a preferential association with Hsp90β over the Hsp90α isoform, resulting in functional Hsp90β-GCUNC-45 interactions that more efficiently block progression of PR chaperoning than seen with Hsp90α-GCUNC-45 complexes (Chadli *et al.*, 2008b). An EEVD-like motif interaction with a TPR domain has also been described for androgen receptor recognition by SGT, where binding is mediated by the first 2 TPR motifs of the SGT TPR domain and the hinge region located between the DNA-binding and ligand-binding domains in the receptor (Buchanan *et al.*, 2007).

Hip has similarly been reported to bind the Hsp70 N-terminal ATPase domain via its TPR domain (Höhfeld *et al.*, 1995). Through this interaction, Hip, originally identified in progesterone receptor complex assembly (Prapapanich *et al.*, 1996a; Smith, 1993), can stabilize substrate-Hsp70 binding and competitively counteract the destabilizing effects of the non-TPR cochaperone BAG1 (Bimston *et al.*, 1998; Gebauer *et al.*, 1997; Höhfeld & Jentsch, 1997; Takayama *et al.*, 1997). The Hip-Hsp70 interaction also allows for the simultaneous association of Hip with Hsp70-Hop complexes (Gebauer *et al.*, 1997; Prapapanich *et al.*, 1996a). By analogy with the mode of GCUNC-45 interaction with Hsp90, there is the possibility that Hip targets a similar TPR recognition site in the N-terminal region of Hsp70. However, Hip is unique among the steroid receptor-associated TPR proteins in terms of Hsp recognition in that it binds Hsp70 independently of EEVD interactions (Höhfeld *et al.*, 1995), and that efficient binding may be due to a greater requirement for additional Hsp-interaction determinants, such as the adjacent highly charged region and a C-terminal DP-repeat domain (Prapapanich *et al.*, 1998). It is possible the mechanism of Hsp70 recognition by Hip is not unique, but may be utilized by some of the steroid-receptor TPR cochaperones to interact with binding partners in distinct cellular pathways. Dutta and Tan (2008) reported the SGT TPR domain is sufficient to bind Vpu and identified the sequence ^{31}KILRQ^{35} in Vpu as being important for this interaction.

4. p23 and Cdc37 interaction with Hsp90

p23 is an essential component involved in stabilizing mature steroid receptor-Hsp90 complexes and binds to the ATP-bound conformation of a Hsp90 dimer characterised by high affinity for client proteins (Ali *et al.*, 2006; Felts & Toft, 2003; McLaughlin *et al.*, 2006;

Richter *et al.*, 2004). Conformational changes that accompany ATP binding promote dimeric interaction between the N-terminal domains of the Hsp90 C-terminal dimer to form distinct binding surfaces for separate p23 molecules, thus further underpinning the ATP-bound conformation (Ali *et al.*, 2006; Karagöz *et al.*, 2010). In a recent model proposed for the Hsp90 cochaperone cycle, entry of an immunophilin cochaperone into an existing client protein-Hsp90-Sti1/Hop-Hsp70 complex forms an intermediate complex important for cycle progression. Conversion of Hsp90 to the closed conformation on ATP and subsequent p23 binding then favours the release of Sti1/Hop (Li *et al.*, 2011).

Cdc37 serves as an adaptor predominantly facilitating protein kinase interaction with Hsp90, although additional client proteins, including steroid receptors have been identified (MacLean & Picard, 2003). Similar to Hop, Cdc37 arrests the Hsp90 ATPase cycle and functions as an "early" cochaperone for the recruitment of protein kinase clients to the Hsp90 machinery. Hsp90 binding maps to the Cdc37 C-terminal region, while kinase interaction occurs via the N-terminal domain (Roe *et al.*, 2004). Hsp90 ATPase activity is coupled to an opening and closing of a molecular clamp generated by the constitutive C-terminal Hsp90 dimer at one end in combination with the ATP-dependent association of the N-terminal domains at the other (Prodromou *et al.*, 2000). A structural view of the Hsp90-Cdc37 complex shows Cdc37 located as a dimer between the N-terminal domains of the clamp, thus preventing their interaction (Roe *et al.*, 2004). With cycle progression, loss of one Cdc37 monomer leads to the formation of a stable $(Hsp90)_2$-Cdc37-kinase complex (Vaughan *et al.*, 2006; Vaughan *et al.*, 2008).

5. Receptor-cochaperone interactions

5.1 Cortisol resistance in New World primates; The key role of FKBP51; Structures of FKBP51 and FKBP52

Analysis of glucocorticoid resistance in New World primates, such as squirrel monkey, has demonstrated that the high circulating cortisol levels result from elevated expression and greatly increased incorporation of FKBP51 into GR-Hsp90 complexes, causing a significant decrease in GR hormone binding affinity (Denny *et al.*, 2000; Reynolds *et al.*, 1999; Scammell *et al.*, 2001). FKBP51 then appears to have a major role in stabilizing an inactive receptor conformation. The FK506 drug-binding pocket of FKBP51 is inaccessible to FK506 in low affinity hormone-binding GR heterocomplexes. However, incubation of receptor cytosols from squirrel monkey lymphocytes with FK506 prevented assembly of FKBP51 with GR-Hsp90 complexes, correlating with a sharp increase in receptor hormone binding and affinity. On the other hand, recognition of FK506 by FKBP52 appeared unaffected by whether the immunophilin exists as a component of mature, high affinity hormone-binding GR complexes or not (Denny *et al.*, 2000; Tai *et al.*, 1992). Furthermore, the immunosuppressant blocks FKBP52-mediated potentiation of GR activity (Riggs *et al.*, 2003). The inhibitory influence of FKBP51 on GR activity requires both FK domains, as well as Hsp90 binding, but is not reliant on FKBP51 PPIase activity (Denny *et al.*, 2005). FK506 may likely serve to sterically hinder receptor LBD interactions with the FK1 domain of FKBP51 and FKBP52 essential for inhibitory and activation effects on receptor, respectively. This differential action of FK506 may arise from distinct domain orientations that have been

defined from recent structures of the two immunophilins (Sinars *et al.*, 2003; Wu *et al.*, 2004). Unique interactions between receptor and the FKBP51 and FKBP52 cochaperones have been further highlighted by results showing that deletion of the Asp195, His196, Asp197 insertion within the FK2 domain of FKBP51 compromised assembly of the immunophilin into PR complexes, whereas removal of the corresponding FK2 insertion loop from FKBP52 had no affect on receptor association (Sinars *et al.*, 2003). This raises the possibility that direct interaction of FK2 in FKBP51 with PR might favour the preferred association of FKBP51 over FKBP52 with this receptor.

5.2 Cortisol resistance in the guinea-pig; Do guinea pig GR LBD changes favour FKBP51 binding over FKBP52?

In contrast to the New World primates, the cause of glucocorticoid resistance in the guinea pig, a New World hystricomorph, has been delineated to an unstructured loop between helix 1 and helix 3 of the guinea pig GR LBD. Five amino acid substitutions in this region differentiate guinea pig GR from the human receptor, with at least four contributing to the low binding affinity phenotype (Fuller *et al.*, 2004). It has been predicted that these crucial residues (Ile538, His539, Ser540, Thr545 and Ser546) lying on the surface of the guinea pig GR LBD, disrupt a contact domain for FKBP52, favouring increased association with FKBP51 and conformational changes that compromise high affinity cortisol binding. Using a yeast-based assay (Riggs *et al.*, 2003) with rat GR substituted in the helix 1 to helix 3 loop with the guinea pig GR-specific residues, we have recently confirmed that FKBP52 can efficiently potentiate the transcriptional activity of the mutated GR, thus discounting a central role of this region in receptor-FKBP52 interaction [Cluning C and Ratajczak T, unpublished observations].

5.3 FKBP52 potentiation of AR, GR and PR

Direct interaction studies between bacterially expressed FKBP52 and GST-tagged, wild type human GR and C-terminal truncation mutants of the receptor purified from Sf9 cell extracts, identified a 35-amino acid region (hGR 465-500), between the DNA-binding domain and the LBD, to be sufficient for FKBP52 binding, with optimal interaction requiring involvement of the LBD (Silverstein *et al.*, 1999). However, recent demonstration of FKBP52 potentiation of GR activity in association with increased receptor hormone binding affinity has definitively localized the FKBP52 effect to the GR LBD (hGR 521-777) and at the same time pointed to a requirement of FKBP52 PPIase activity residing in the FK1 domain (Riggs *et al.*, 2003). Studies with FKBP52 knockout mouse strains have extended the critical physiological role of FKBP52 to cellular responses controlled by both AR (Cheung-Flynn *et al.*, 2005) and PR (Tranguch *et al.*, 2005; Yang *et al.*, 2006), while similar influences of this immunophilin cochaperone on ERα (Riggs *et al.*, 2003) and MR (Gallo *et al.*, 2007) activity have not been observed, despite the assembly of FKBP52 with Hsp90 complexes containing these receptors.

5.4 Molecular basis of FKBP52 action; Potential interaction of FKBP52 with the BF3 regulatory site

An initial understanding that FKBP52 potentiation of AR, GR and PR activity was dependent on the FK1-mediated PPIase function of the immunophilin, prompted speculation that FKBP52

might target a key proline likely to be conserved among these receptors and that this critical residue would be located on the surface of the LBD, accessible to the cochaperone and in a position where it might influence the shape of the ligand binding pocket (Cheung-Flynn *et al.*, 2005). Although several such candidate prolines exist in the intervening loops between receptor LBD helices, a more extensive mutational analysis of the FK1 catalytic site has excluded a role for the FKBP52 PPIase activity in receptor potentiation (Riggs *et al.*, 2007). Rather, recent evidence has identified a loop overhanging the FK1 catalytic pocket in FKBP52 that is responsible for the functional difference between FKBP52 and FKBP51 relating to AR (and GR/PR) potentiation (Riggs *et al.*, 2007). It is proposed that a critical proline within this loop (human FKBP52 Pro119) allows specific contact with a region of the AR LBD (a structural feature that is also common to GR and PR), thus helping to stabilize an LBD conformation favourable for high affinity hormone binding and leading to efficient transcriptional activation (Riggs *et al.*, 2007). It is speculated that a leucine substitution within the corresponding FK1 sequence of FKBP51 alters the loop conformation sufficiently to disrupt this functionally important contact. The possibility exists that in the hormone-induced transition from inactive to active states of AR-Hsp90 complexes associated with FKBP51 and FKBP52, respectively, Hsp90 orients FKBP52 to achieve unique interactions with the receptor LBD, allowing Hsp90 to facilitate optimal hormone binding and to further fine-tune the hormonal response.

Prior to investigations establishing a noncatalytic involvement of the FKBP52 PPIase domain in the modulation of receptor function, an early attempt to identify the putative proline substrate for FKBP52 isomerase activity within the AR LBD utilized AR-P723S, a proline mutant associated with androgen insensitivity syndrome (Cheung-Flynn *et al.*, 2005). Although predicted to display basal activity, coupled with a lack of response to hormone in the presence of FKBP52, this mutant was characterized by subnormal activity in the absence of FKBP52, showing full restoration to wild type receptor activity levels with the cochaperone on exposure to hormone (Cheung-Flynn *et al.*, 2005). Such a favoured response reflects a greater dependence of the AR-Pro723S mutant on FKBP52 for normal activity. Pro723 lies within the signature sequence conserved among all steroid receptors (Brelivet *et al.*, 2004), close to a region directly involved in ligand binding and is situated in a solvent exposed loop between helices 3 and 4, which combine together with the mobile helix 12 to form the AF2 coactivator binding pocket (He *et al.*, 2004; Matias *et al.*, 2000b). For AR, AF2 initially has a preferred interaction with the AR N-terminal domain, resulting in an intramolecular fold that precedes receptor dimerization and appears critical for AR function (He *et al.*, 2001; He *et al.*, 2004; Schaufele *et al.*, 2005). Pro723 also forms part of the recently identified BF-3 surface that has the ability to allosterically alter the AF2 binding pocket of AR (Estébanez-Perpiñá *et al.*, 2007) (Fig. 4). BF-3 residues altered through natural mutations linked to androgen insensitivity and those associated with prostate cancer, either diminish or enhance AR AF2 activity, respectively, underlining the importance of the BF-3 surface for AR function (Estébanez-Perpiñá *et al.*, 2007). FKBP52 rescue of AR-Pro723S activity might signify FKBP52 influence over some part of the BF-3 allosteric regulatory site leading to conformational changes that allow full recovery of AR activity. Indeed, Cox and coworkers have recently identified small-molecule inhibitors of FKBP52-enhanced AR function in prostate cancer cells that target a region of the AR LBD overlapping the BF3 surface (De Leon *et al.*, 2011) (Fig. 4). Multiple residues that contribute to the FKBP52 sensitivity of AR, some of which form part of the binding site for MJC13, the lead compound, have been

identified (De Leon *et al.*, 2011) (Fig. 4). Since MJC13 helps to maintain an intact AR-Hsp90-FKBP52 complex at low hormone concentrations, it is possible that the inhibitor interferes with a critical next step - a hormone-induced, FKBP52-dependent transitory change in AR conformation necessary for nuclear translocation. Sequence comparisons have revealed some conservation of BF-3 residues within the LBDs for AR, GR, MR and PR, suggesting the presence of BF-3-like regulatory domains in each receptor (Estébanez-Perpiñá *et al.*, 2007) (Fig. 4). A very limited conservation of these residues is apparent in ERα, suggesting the formation of a BF-3 type surface that is unique to this receptor (Estébanez-Perpiñá *et al.*, 2007) (Fig. 4). Both ERα and MR behave differently to AR, GR and PR, through their inability to respond to FKBP52. Certain structural differences within their LBDs distinguish these two receptors from the other members of this subfamily (De Leon *et al.*, 2011) (Fig. 4). Since FKBP52 also regulates GR and PR activity, most likely through specific BF3 surfaces, there is the potential for the development of FKBP52-specific inhibitors targeting GR and PR function to treat a range of steroid hormone-based diseases (Moore *et al.*, 2010). The BF-3 pocket is a potential target for second-site modulators that can allosterically block agonist-activated AR function to inhibit prostate cancer cell growth (Joseph *et al.*, 2009).

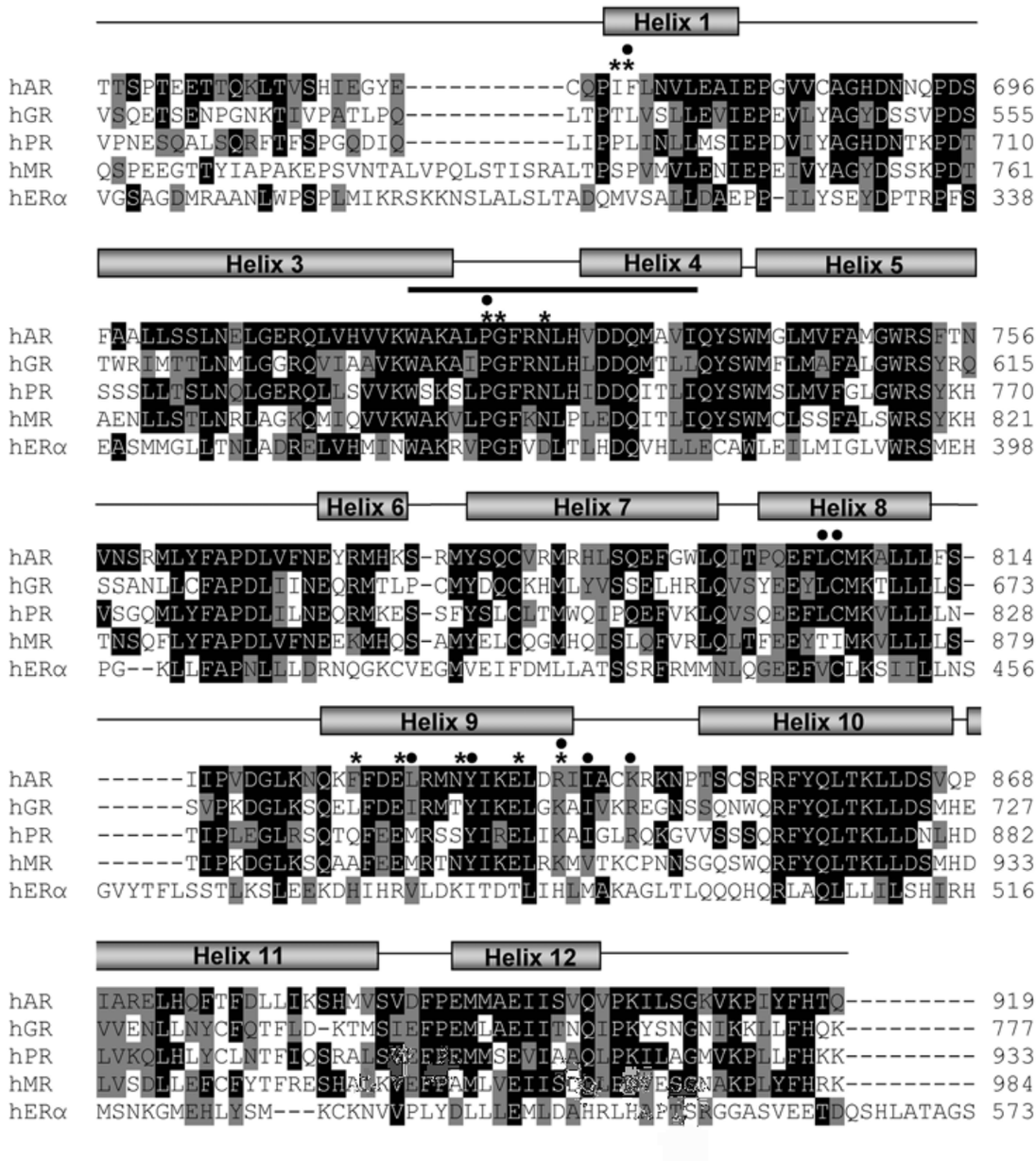
5.4.1 FKBP51 is an androgen-regulated gene that promotes assembly of mature AR-Hsp90 complexes

FKBP51 is recognised as a highly sensitive AR-regulated gene that functions as an important component of a feed-forward mechanism linked to the partial reactivation of AR-signalling pathways in the absence of androgens, leading to the outgrowth of androgen-independent tumours (Amler *et al.*, 2000; Febbo *et al.*, 2005; Magee *et al.*, 2006; Mousses *et al.*, 2001; Tomlins *et al.*, 2007). Sanchez and coworkers have confirmed a significantly increased expression of FKBP51, but not that of FKBP52, in most prostate cancer tissues and in androgen-dependent and androgen-independent cell lines (Periyasamy *et al.*, 2010), suggesting that FKBP51 might have a critical role in prostate cancer growth and progression. FKBP51 overexpression was found to increase the AR transcriptional response by facilitating hormone-binding competence through the assembly of the AR LBD with mature FKBP51-Hsp90-p23 complexes (Ni *et al.*, 2010), resulting in higher levels of androgen-liganded receptor and providing a pathway for AR-dependent signalling and growth in a low-androgen environment. The ability of FKBP51 to enhance AR transcription and chaperone complex assembly appears to be dependent on FKBP51 PPIase activity mediated by the FK1 domain and requires Hsp90 binding through its TPR domain (Ni *et al.*, 2010).

6. Receptor LBD contacts with other Hsp90 cochaperones

6.1 PP5; GCUNC-45; SGT

The domain structure of the Hsp90 cochaperone, PP5, a serine/threonine protein phosphatase (Chen *et al.*, 1994; Chinkers, 1994), is characterised by a C-terminal phosphatase catalytic domain and an N-terminal TPR domain that competes with FKBP51, FKBP52 and CyP40 for the TPR binding site at the Hsp90 C-terminus during assembly into mature steroid receptor-Hsp90 complexes (Banerjee *et al.*, 2008; Chen *et al.*, 1996a; Hinds Jr & Sanchez, 2008). Through its TPR domain, PP5 has also been shown to bind directly to ERα and ERβ, an interaction that targets the LBDs of these receptors, but does not require the C-terminal region incorporating



NCBI accession numbers for receptor sequences are: AR – NP000035, ERα – NP000116, GR – NP001018087, MR – NP000892, PR – NP000917. The ERα sequence has 595 amino acids and is shown terminated at residue 573. LBD helices are based on the structure of AR liganded to R1881 (Matias *et al.*, 2000a) (PDB ID 1E3G). The nuclear receptor signature sequence is indicated (thick black line). Residues that map to the BF-3 allosteric regulatory site defined for AR are highlighted with an asterisk (*). Multiple residues that contribute to the FKBP52 sensitivity of AR and form the putative binding site for MJC13 (De Leon *et al.*, 2011) are highlighted with a black circle (•). Identical residues are shown white against black; conserved residues (black on grey) are based on the following scheme: (P, G), (M, C), (Y, W, F, H), (L, V, I, A), (K, R), (E, Q, N, D) and (S, T).

Fig. 4. Multiple sequence alignment of human steroid receptor LBDs.

the helix 11-12 loop and helix 12 central to AF2 function (Ikeda *et al.*, 2004). PP5 was found to function as a negative regulator of ERα transcription *in vivo* by inhibiting epidermal growth factor (EGF)-dependent phosphorylation of Ser118 in the receptor N-terminal domain. Although demonstration of a direct PP5-ERα interaction was consistent with a non-involvement of Hsp90, a role for this major molecular chaperone in the *in vivo* effects of PP5 on ERα function cannot be discounted. Similar observations have been reported for GR with evidence suggesting that PP5-dependent modulation of receptor N-terminal phosphorylation within the GR-Hsp90 apo-receptor complex is mediated through contacts between the phosphatase and receptor LBD (Wang *et al.*, 2007).

A yeast two-hybrid screen, using bait encompassing both the hinge region and LBD of human PR, liganded with the mixed antagonist RU486, identified GCUNC-45 as a PR-binding protein (Chadli *et al.*, 2006). Presence of two LXXLL motifs (similar to NR boxes of known transcriptional coregulatory proteins) within the interacting clone, corresponding to the C-terminal end of GCUNC-45, suggested a mode of interaction similar to that for receptor recognition of transcription coactivators (Ratajczak, 2001), although this remains to be confirmed. Both FKBP52 and CyP40 compete with GCUNC-45 for the N-terminal TPR site, with nucleotides causing a reduction in Hsp90 binding affinity for these cochaperones in this region and favouring their interaction with the Hsp90 C-terminus during progression of receptor to a hormone-binding state (Chadli *et al.*, 2008b). GCUNC-45 therefore, appears to have a role upstream of FKBP52 and CyP40, at an intermediate stage of the receptor activation pathway.

The Hsp70/Hsp90 cochaperone, SGT, has been shown to interact through its TPR domain with the hinge region of human AR, which contains a peptide sequence structurally resembling the EEVD binding site for TPR proteins at the extreme C-terminus of Hsp70 and Hsp90 (Buchanan *et al.*, 2007). It has been proposed that, as a component of AR-Hsp90 complexes, SGT regulates the ligand sensitivity of AR signalling by limiting receptor trafficking to the nucleus at low hormone concentrations and maintaining the receptor within the cytoplasm of the cell.

6.2 p23; Cdc37

Disruption of the p23 gene in mice has revealed that although p23 is not essential for overall perinatal development its absolute requirement for perinatal survival is linked to impaired GR function arising most likely from instability of GR-Hsp90 complexes in the absence of p23 (Grad *et al.*, 2006; Picard, 2006). These findings suggest that GR might be a key molecular target for p23. Overexpression experiments with p23 in tissue culture cells have revealed both positive and negative influences on GR function (Freeman *et al.*, 2000; Wochnik *et al.*, 2004), as well as differential effects on other steroid receptors - increasing PR activity, while decreasing the activities of AR, ERα and MR (Freeman *et al.*, 2000). In yeast, p23 has been shown to be a positive regulator of ERα transcriptional activation, being most effective at low ERα levels and hormone concentrations, consistent with the proposed role for p23 as a component of mature ERα-Hsp90 complexes (Knoblauch & Garabedian, 1999). Ectopic expression of p23 in MCF-7 breast cancer cells increased both hormone-dependent and hormone-independent ERα transcriptional activity (Knoblauch & Garabedian, 1999).

Thus, while the major impact of p23 on ERα is likely to be through an Hsp90-dependent effect on estradiol binding, p23 overexpression may also influence receptor activity independent of ligand binding and may participate in the disassembly of receptors at cognate response elements (Freeman *et al.*, 2000; Freeman & Yamamoto, 2001; Freeman & Yamamoto, 2002). It is of interest that although p23 increases AR transcriptional activity in a variety of mammalian cell lines, partly by increasing ligand binding competence of the receptor, Hsp90 inhibitors could not abolish the AR coactivation potential of p23, consistent with an Hsp90-independent role of p23 in AR function (Querol Cano L and Bevan CL, unpublished observations).

Genetic studies in yeast have revealed that Cdc37 plays a role in AR hormone-dependent transactivation through functional interactions with the AR LBD, although the hormone-binding properties of the receptor appear to be unaffected (Fliss *et al.*, 1997). The association with Cdc37 is specific to AR since it does not occur with closely related nuclear receptors such as GR (Rao *et al.*, 2001). Depletion of Cdc37 using RNA interference caused growth arrest in both AR-positive and AR-negative prostate cancer cells, and in the former led to a loss of AR transcriptional activity with a concomitant decrease in androgen-dependent gene expression (Gray *et al.*, 2007). The targeting of Cdc37 in prostate cancer causes growth inhibition that correlates with decreased signalling through multiple pathways - the extracellular signal-regulated kinase (ERK) and Akt kinase cascades, as well as reduced AR-dependent signalling (Gray *et al.*, 2008).

7. Conclusions

We have arrived at a better understanding of the molecular mechanisms that allow the Hsp90 chaperone to modulate steroid receptor function through direct contact with receptor LBDs. Critical to this regulation is the ability of Hsp90 to coordinate and bring to receptor-Hsp90 complexes a selection of cochaperones whose specialized influences target receptor LBDs and combine, at various stages of the receptor activation pathway, to alter receptor hormone-binding status, cellular location and transcriptional activity. A number of these cochaperones may impact on steroid receptor function independently of Hsp90. Substantial gaps still remain, however in our knowledge of how the interplay between Hsp90 and its cochaperones affects receptor function. For example, while it is known the CyP40 yeast homologue, Cpr6, regulates Hsp90 ATPase activity during receptor assembly (Prodromou *et al.*, 1999) and studies of a second yeast homologue, Cpr7, have provided some insight into the role of this immunophilin in Hsp90-dependent signalling by steroid receptors (Duina *et al.*, 1996; Duina *et al.*, 1998), a coherent mechanism at the molecular level has yet to be defined. From the structural similarity between CyP40 and FKBP52, both being characterized by N-terminal PPIase and C-terminal TPR domains, it is tempting to draw parallels for their mechanism of action. Within steroid receptor-Hsp90 complexes it is possible that, as for FKBP52, the CyP40 PPIase domain forms productive interactions with the receptor LBD, serving to modulate receptor conformation and function. This may be of relevance for the function of ERα, purification of which led to the isolation of CyP40 in ERα-Hsp90 complexes (Ratajczak *et al.*, 1993) and for the regulation of AR in prostate cancer where CyP40 appears to be overexpressed (Periyasamy *et al.*, 2010).

Hsp90 is required for the proper function of several key regulatory proteins including multiple tyrosine and serine/threonine kinases and steroid receptors, many of which are involved in promoting malignancy (Calderwood *et al.*, 2006; Pearl, 2005; Whitesell & Lindquist, 2005). The aim of targeting and pharmacological manipulation of the Hsp90 chaperoning system has led to the ongoing development and clinical evaluation of novel Hsp90 and chaperone inhibitors for potential application in therapies against selected malignancies (Donnelly *et al.*, 2010; Kim *et al.*, 2009), syndromes arising from dysfunctional protein folding and neurodegenerative diseases (Jinwal *et al.*, 2010). With growing understanding of the novel mechanisms through which Hsp90 cochaperones modulate the function of specific clients, strategies are now evolving for the targeting of chaperone-client interactions in a wide range of human diseases (De Leon *et al.*, 2011; Gray *et al.*, 2008).

8. Abbreviations

Hsp, heat shock protein; TPR, tetratricopeptide repeat; PPIase, prolylpeptidyl isomerase; FKBP, FK506-binding protein; CyP40, cyclophilin 40; PP5, serine/threonine protein phosphatase type 5; GCUNC-45, general cell UNC-45; αSGT, small glutamine-rich tetratricopeptide repeat containing protein α; AR, androgen receptor; ERα, estrogen receptor α; ERβ, estrogen receptor β; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; LBD, ligand-binding domain; AF2, activation function 2; GST, glutathione S-transferase.

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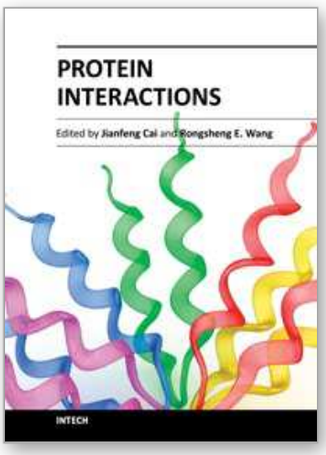

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Protein interactions, which include interactions between proteins and other biomolecules, are essential to all aspects of biological processes, such as cell growth, differentiation, and apoptosis. Therefore, investigation and modulation of protein interactions are of significance as it not only reveals the mechanism governing cellular activity, but also leads to potential agents for the treatment of various diseases. The objective of this book is to highlight some of the latest approaches in the study of protein interactions, including modulation of protein interactions, development of analytical techniques, etc. Collectively they demonstrate the importance and the possibility for the further investigation and modulation of protein interactions as technology is evolving.

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