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Review

Immunophilins: for the love of proteins

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Abstract. Immunophilins are chaperones that may also exhibit peptidylprolyl isomerase (PPIase) activity. This review summarizes our knowledge of the two largest families of immunophilins, namely cyclophilin and FK506-binding protein, and a novel chimeric dual-family immunophilin, named FK506- and cyclosporin-binding protein (FCBP). The larger members of each family are modular in nature, consisting of multiple PPIase and/or protein-protein interaction domains. Despite the apparent difference in their sequence and three-dimensional structure, the three families encode similar enzymatic and biological functions. Recent studies have revealed that many immunophilins possess a chaperone function independent of PPIase activity. Knockout animal studies have confirmed multiple essential roles of immunophilins in physiology and development. An immunophilin is indeed a natural 'protein-philin' (Greek 'philin' = friend) that interacts with proteins to guide their proper folding and assembly.

Keywords. Immunophilin, PPIase, chaperone, cyclophilin (Cyp), FK506-binding protein (FK506), trigger factor, TPR domain, dual-family immunophilin, protein folding.

Introduction

The immunophilin superfamily consists of highly conserved proteins, many but not all of which possess peptidylprolyl cis-trans isomerase (PPIase) or rotamase activity [1]. They belong to two major sequence families that bind specific immunosuppressant molecules of fungal origin: the cyclophilins (Cyps) bind cyclosporin A (CsA), a cyclic undecapeptide, while the FK506-binding proteins (FKBPs) bind macrolides such as FK506 (tacrolimus) and rapamycin (sirolimus) that are structurally unrelated to CsA. The drugs bind to the catalytic pocket of the PPIase domain and inhibit the PPIase activity. Additionally, the drug-immunophilin complexes, Cyp-CsA and FKBP-FK506, dock on to protein phosphatase 2B (PP2B) (also known as calcineurin), thereby inhibiting the phosphatase activity of PP2B [2, 3]. This results in the elevated phosphorylation of a number of PP2B substrates, including nuclear factor of activated T cells (NF-AT). Phospho-NF-AT fails to translocate to the nucleus, and as a consequence immunologically important genes in the T cell remain silent, suppressing the immune reaction. This class of drugs is indeed widely used in the clinic to suppress rejection of donor organs in recipients. Rapamycin, a highly potent fungicide, immunosuppressant and anticancer agent, functions differently. The rapamycin-FKBP complex does not inhibit PP2B but, instead, inhibits a protein named target of rapamycin (TOR), a Ser/Thr protein kinase essential for a variety of pathways [4]. However, these drug-dependent functions, which gave rise to the 'immunophilin' name, have only clinical but no physiological relevance, since healthy mammalian cells never naturally encounter the immunosuppressant drugs.

Biochemical and sequence analyses have led to the identification of a large number of Cyp and FKBP homologs in various organisms [1]. In addition, a novel family of chimeric immunophilins has recently been identified that contain both Cyp and FKBP sequences [5]. Due to their dual nature, these latter enzymes are named FCBPs (for FK506- and CsA-binding proteins). In general, the

nomenclature of the immunophilins uses the genus and species initials as prefix and the predicted molecular weight in kDa as suffix; thus HsCyp18 is *Homo sapiens* cyclophilin, 18 kDa. However, old names never seem to die and are used interchangeably; thus, Cyp18 is also called CypA.

This review is not meant to be an encyclopedia of the rapidly growing number of immunophilins; rather, the goal is to analyze a few representative examples in order to offer a mechanistic framework of their structure and function with special emphasis on protein folding. The main focus is on the natural role of immunophilins and not on the drug-dependent immunosuppressive properties.

PPIase activity

The planar peptide bond in proteins is predominantly in the trans conformation [6]; however, proline (Pro) residues are unique in that they have a relatively high intrinsic probability of being in cis. The trans-cis isomerization of the peptide bonds on the N-terminal side of Pro is thus essential for proper protein folding of the nascent protein. This is a rate-limiting process that is accelerated by enzymes with PPIase activity such as the immunophilins [7, 8]. Historically, although the first Cyp was identified in 1984 by its affinity purification via a CsA column [9], not until 1989 was its PPIase activity identified [10, 11].

Since then, Cyps and FKBPs have been shown to influence the folding of a number of synthetic peptides and natural proteins such as collagen [12–14], chymotrypsin inhibitor [15], carbonic anhydrase [16, 17], and ribonuclease [18]. However, the exact catalytic mechanism of the PPIase activity remains hazy, and there is still no convincing evidence that the PPIase activity of immunophilins is absolutely essential *in vivo*. As elaborated later, immunophilins can indeed chaperone and guide proteins in ways that are independent of their PPIase activity.

PPIase domains: conserved enzymatic activity despite different sequence

An interesting feature of immunophilins is that although both Cyp and FKBP families possess PPIase activity, the sequence and structure of the two families are dissimilar (Figs. 1–3) [19, 20]. CypA contains eight anti-parallel β strands that form a right-handed β barrel with an α helix at either end (Fig. 3). However, CypA is a uniquely closed β barrel so that neither CsA nor the Pro-containing substrate peptide can bind to the hydrophobic core, suggesting that it is not evolutionarily related to other β barrel structures. CsA and peptides instead bind to the outer surface of CypA, although a number of critical hydrophobic residues are involved in binding (Fig. 3). FKBP12, in contrast, is composed of five β strands wrapping around a short α helix with an overall conical shape that has a hydrophobic groove to which FK506 and PPIase substrates bind.

Though the structures of CypA and FKBP12 differ, in both proteins the substrate and the inhibitory immunosuppressants compete for binding to the PPIase active site. Thus, the 'PPIase domain' has become synonymous with 'drug-binding domain', i.e. cyclosporin-binding domain (also known as CLD) in Cyp, and FK506-binding domain (FKBD) in FKBP [1]. The rest of the immunophilin sequence often plays a family-specific role,

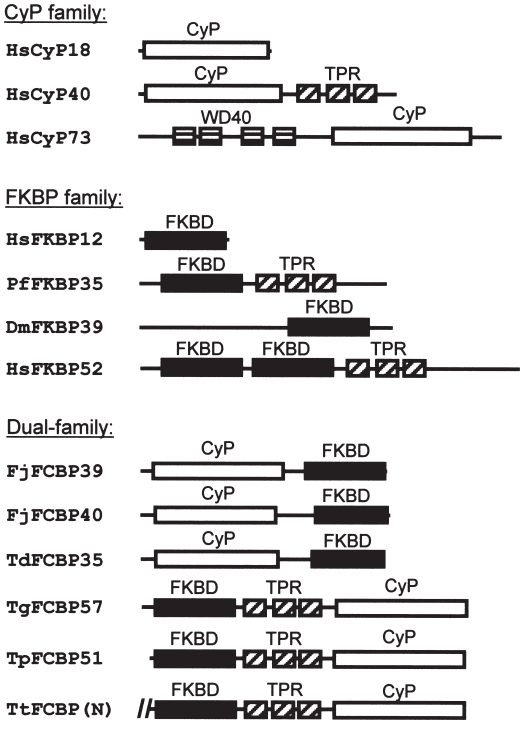

Figure 1. Representative domain arrangements of immunophilins [5, 30, 31]. The single-family enzymes (FKBP, CyP) are contrasted with the newly discovered dual-family members. The proteins are named by the initials of the organism (Pf, *Plasmodium falciparum*; Dm, *Drosophila melanogaster*; Fj, *Flavobacterium johnsonii*; Hs, *H. sapiens*; Td, *Treponema denticola*; Tg, *Toxoplasma gondii*; Tp, *Theileria parva*; Tt, *Tetrahymena thermophila*), followed by the domain name(s) and the theoretical molecular weight in kDa. The *Tetrahymena* enzyme, as predicted from the draft genome sequence, has an unusual N-terminal extension and, thus, its size remains uncertain (denoted by N). The accessory domains, i.e. TPR (tetratricopeptide repeat), WD40 (a roughly 40 amino acid-long conserved sequence containing a Trp-Asp dipeptide at its C terminus) and the putative CaM (calmodulin)-binding domains are indicated. Cyp domains are in black and FKBDs are white. Due to their small number, all six FCBP sequences found to date are shown [5; our unpublished data]. The NCBI accession numbers are: HsCyP18, AAU13906; HsCyp40, NP_005029; HsCyp73, BAA07555; HsFKBP12, NP_000792; PfFKBP35, NP_701815; DmFKBP39, CAA86996; HsFKBP52, AAA36111; FjFCBP39, AAM92026; FjFCBP40, AAM92027; TdFCBP35, NP_972990; TgFCBP57, AAX51680; TpFCBP51, XP_765469.1; TtFCBP(N), EAR86074.1.

Figure 2. Conserved PPIase domains of selected Cyp and FKBP family members. Identical residues (*) and conservative replacements (:) are indicated. The three TPRs in the C-terminal extension of human Cyp40 are marked by long underlines. In both families, the drug-binding residues are shaded gray and the consensus substrate-binding residues are underlined (37, 38, 99–103). In CypA, the guanidinium group of the invariant R55 plays a particularly critical role in PPIase function by anchoring the substrate Pro oxygen and stabilizing sp3 hybridization of the Pro nitrogen in the transition state [104]. Note the lack of any obvious similarity between functional residues of the two families, although their enzymatic activity (i.e. PPIase) is conserved. The NCBI accession numbers are: *Leishmania donovani* Cyp (LdCyp), AAD46565; PfCyp19A, CAB39039; HsCyp40, NP_005029; the rest are given in Figure 1.

such as interacting with specific subsets of client proteins, but may also directly contribute to protein folding (see later).

This is reminiscent of the strategy adopted by a number of other protein families, such as protein Ser/Thr phosphatases (PPs) [21], whereby all members of the PP superfamily catalyze the same basic reaction of dephosphorylating P-Ser and P-Thr residues. However, whereas the phosphatases of the PPP class share a highly conserved sequence and structure in their catalytic core [21, 22], the metal-dependent PPM phosphatases have a very different sequence and a novel protein fold [23]. Finally, larger immunophilins and phosphatases contain protein-protein interaction domains outside the catalytic core, such as the tetratricopeptide repeat (TPR) domains, which allosterically modulate the activity of these proteins (see below) [24, 25].

Overall domain architecture of immunophilins

All higher organisms possess multiple Cyp and FKBP paralogs [1, 26]. Humans, for example, have at least 16 Cyps and 15 FKBPs that exhibit a range of molecular weights and intracellular locations. The prototypical minimal Cyp, HsCypA (Cyp18), contains just the PPIase domain (Fig. 1, 2); this Cyp and the HsCyp40 reside in the cytosol. Others may possess signal sequences that target them to specific organelles. Four small Cyps are located in the following organelles: HsCypB in the endoplas-

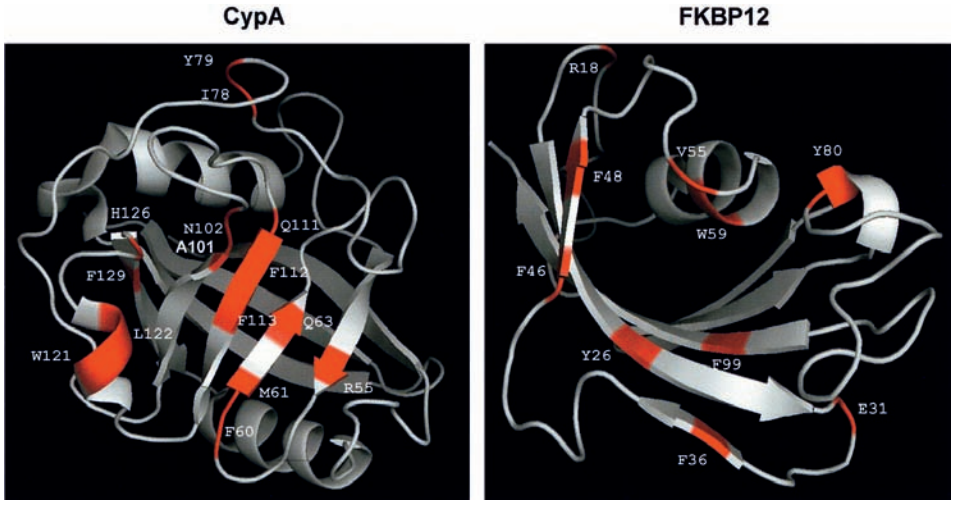

Figure 3. The binding surface of the PPIase domain in immunophilins. In this 'ribbon' presentation of the smallest members of each family, CypA and FKBP12, the invariant amino acids of the PPIase domains involved in substrate-binding (underlined in Figure 2) are colored red and labeled. The structures are oriented for optimal visibility of these residues to the reader. Note that the majority of the residues are hydrophobic and form a flexible and accessible domain to accommodate a variety of client proteins. The structures were drawn using PyMOL v0.99 (http://pymol.sourceforge.net/) and PDB co-ordinates from the NCBI structure entries (2CPL and 1FKK, respectively).

mic reticulum (ER), HsCypC in membrane, HsCypD in mitochondria and HsCyp19 in the nuclear spliceosome. Essentially all major human immunophilins have apparently equivalent homologs in other higher eukaryotes and plants. The genome of the lower eukaryote, *Saccharomyces cerevisiae*, also encodes multiple immunophilins, specifically, eight Cyps and four FKBPs, many of which have human counterparts.

Large immunophilins contain additional accessory domains, positioned at various locations relative to the PPIase-like domain [1]. In three large human Cyps, for example, the CLD/PPIase domain is followed by accessory domains: Ser-Arg-Lys-rich domains in Cyp88 and Cyp157, and a TPR domain in Cyp40 (Fig. 1). In three others, the CLD/PPIase domain is preceded by accessory domains: the RNA-binding domain in Cyp33, WD repeats in Cyp73 (Fig. 1) and various domains in Cyp324. The record for the largest Cyp is held by HsCyp358 [1], a Ran-binding constituent of nuclear pores, also called RanBP2 or Nup358. It contains a Leu-rich domain, a Zn-finger, multiple Ran-binding domains, and a C-terminal Cyp domain.

A similar variety of accessory domains is also found in FKBPs (Fig. 1). As with the Cyps, the smallest member, FKBP12, contains a single FKBD/PPIase domain and little else, while the larger FKBPs contain TPR [1, 27–29]. Like Cyps, many FKBPs exhibit specific intracellular locations, such as cytoplasmic, nuclear or ER. No Cyp with a multiple CLD has been found.

Even a cursory glance at Figure 1 reveals that the large immunophilins are composed of modular domains that are functionally independent. This is confirmed by various lines of experimental evidence as follows. In deletion experiments, loss of the TPR motifs, singly or together, had no effect on the PPIase activity of PfFKBP35 *in vitro* [30, 31]. Further evidence for the modular nature of immunophilins is provided by the recent discovery of an extremely novel class of immunophilins in lower organisms [5]. These immunophilins are dual-family proteins that contain both CLD and FKBD-like PPIase domains (Fig. 1). For this reason, it was decided that these enzymes be named FCBP (FK506- and CsA-binding protein). So far, FCBPs have been found only in three protozoan parasites of the *Toxoplasma*, *Theileria* and *Tetrahymena* genera, and in two bacteria, *Flavobacterium johnsonae* and *Treponema denticola* [5]. Although still a relatively minor class, new FCBP homologs are coming to light as more genomes are being sequenced (Fig. 1). Detailed biochemical studies of recombinant *Toxoplasma gondii* FCBP57 showed that both domains possessed PPIase activity with family-specific drug sensitivity, i.e. sensitivity to CsA and FK506, respectively. In the parasitic enzymes, the two PPIase domains are joined by TPR domains that share significant sequence similarity and conserved spacing between the motifs. Deletion of any number of the TPR motifs had no effect on the PPIase activity of Tg-FCBP57. In agreement with the lower evolutionary status of the prokaryotes, the bacterial FCBP homologs are smaller (Fig. 1) mainly because the CLD and FKBD domains are directly joined with no TPR in between, further suggesting that the TPR domains only serve in an accessory role, perhaps to recruit the client proteins.

It is not clear why some large FKBPs have evolved to contain multiple PPIase homology domains. Perhaps an explanation lies in the fact that they are non-identical in structure or function. For example, although FKBP51 and

FKBP52 have two FKBD homology domains (Fig. 1), the first FKBD possesses a topology similar to FKBP12 whereas the second is substantially deviant, partly due to insertions in loop regions, which may sterically exclude the substrates. As a result, it is only the first FKBD that exhibits PPIase catalytic activity [29], suggesting that the second may have a PPIase-independent chaperone function [32]. Moreover, loss of each domain has a distinctive biochemical defect [33], suggesting functional specificity of each. In the dual-family TgFCBP57, both domains have PPIase activity [5] and, thus, may promote proline isomerization in their respective, group-specific clients. It is possible that in these multi-domain immunophilins, the two domains recruit family-specific client proteins in a concerted or simultaneous fashion that would not have been possible if the domains were on separate polypeptides. Perhaps, being present in cis, the two domains can simultaneously chaperone two subunits of a multimeric complex in the appropriate order. When there is a TPR between them (as in TgFCBP57), it may facilitate or stabilize such recruitment by providing additional contacts and also act as a flexible, spring-like hinge to allow an optimal fit of both domains around complexes of different sizes and shapes. The corollary is that the TPR-free multi-domain immunophilins such as FKBP51/52 and the bacterial FCBPs may be less versatile in their choice of clients but still adequate for smaller complexes.

Interestingly, although the small FKBPs contain single PPIase domains, some may dimerize, often promoted by ligands. Detailed studies of the 245 amino acid-long *Escherichia coli* FkpA have recently provided interesting insights [34–36]. Whereas the N-terminal half of FkpA promotes dimerization and exhibits chaperone activity, the C-terminal half belongs to the FKBP family, exhibits PPIase activity and binds FK506. Dimerization places the two active sites of the FKBP domains in juxtaposition, such that they can simultaneously interact with a protein, and this is required for full catalytic activity [35]. In essence, such a dimer can be viewed as a multi-domain immunophilin in which the two homo-domains are symmetrically presented in trans.

PPIase activity versus chaperone function

Despite the documented need of immunophilins in biology, the contribution of their PPIase activity has been debated [1, 37–40]. Specific inhibition of PPIase activity by immunosuppressant drugs excluded a role for PPIase catalytic activity in chaperone function [41, 42]. Of the two yeast Cyp40 paralogs, Cpr6 has greater PPIase activity whereas Cpr7 is a more active chaperone [43]. *Arabidopsis thaliana* FKBP42, another large immunophilin, contains a PPIase homology domain followed by TPR (resembling the PfFKBP35 domain structure; Fig. 1), but it is neither a PPIase nor capable of binding FK506. Recent crystal structure analysis of AtFKBP42 provides a basis for this defect. A number of critical residues that form the hydrophobic cleft of a functional PPIase in FKBP (Fig. 2, 3) are either missing in AtFKBP42 or oriented in a different direction due to insertions in loop regions [44]. The insertions, in addition, are postulated to block the access of substrates to the cleft. Overall, this is reminiscent of the inactive, second FKBD in FKBP51/52 and confirms that at least a subset of chaperone function of AtFKBP42 is indeed PPIase independent.

Many prokaryotes do not contain any FKBP or Cyp. However, some encode a unique family of ribosome-associated PPIases known as trigger factor (TF), which aid the folding of nascent polypeptide chains on ribosomes [45]. *In vitro* the refolding yields of guanidine-denatured bovine carbonic anhydrase II, assisted by wild type TF and by mutants lacking PPIase activity, are about the same, providing experimental evidence that the PPIase and chaperone activities of TF are independent [46–48]. Site-directed mutagenesis of F198 of TF, which is equivalent to W60 of the PPIase domain of FKBP12 (Fig. 1, 2), destroys the PPIase activity while retaining the chaperone function *in vitro* and *in vivo* [48]. Further evidence for PPIase-independent chaperone functions derives from attempts to map these domains, as described below.

Although the exact domains or residues responsible for the chaperone function per se remain unclear, a role of novel hydrophobic grooves (outside the PPIase binding surface in Figure 3) is becoming increasingly apparent. Deletion analysis has shown that in the large immunophilins, FKBP51/52, only the first domain has PPIase activity. In contrast, the chaperone activity resides mainly within the C-terminal part that includes the TPR region, which also shows its separation from the enzymatic activity [29]. Recently, further insights into the elusive chaperone groove were gained from deletion analysis of Cyp40 that contains a PPIase domain linked to TPR (Fig. 1) [49]. These results show that the PPIase domain plus linker or the linker region plus TPR domain retains chaperone activity, while individually, neither the catalytic nor the TPR domains had chaperoning ability. Thus, the Cyp40 chaperone function appears to be localized within the linker that forms a potential protein-binding cleft [49].

Recent experiments have shown that even the small immunophilins containing very little sequence besides the PPIase domain may have a chaperone function distinct from the PPIase activity. PPIase-independent chaperone function of FkpA, a small periplasmic chaperone of *E. coli*, has been discussed earlier [34–36]. The 17- to 18-kDa 'short-type' archaeal FKBP from *Methanothermococcus thermolithotrophicus* shows a PPIase activity and a chaperone-like activity capable of refolding proteins and suppressing aggregation [50]. Mutational analysis revealed that the chaperone-like activity is independent of

the PPIase activity. The single-domain, 187 residue-long LdCyp from *Leishmania donovani* is able to disaggregate soluble aggregates of a client protein even when a large portion of the N-terminal PPIase domain is deleted [51]. Homology modeling of the truncated LdCyp that retains folding activity also reveals a hydrophobic crater that is normally buried inside the polypeptide but becomes solvent exposed due to the deletion [51]. However, this alternate crater is different from the hydrophobic groove in the full-length protein.

Unfortunately, no structural studies have yet been done for larger protein clients in complex with immunophilins to validate these novel binding grooves. Three-dimensional structures are only available for co-complexes of small Pro-containing peptides with immunophilins, which reveal that the peptides bind essentially the same PPIase domain where the immunosuppressant drugs also bind (for example, compare NCBI 1FGL, structure of CypA complexed with a fragment of HIV-1 Gag protein, with 3CYS, structure of CypA complexed with CsA). In any case, the binding surface of all immunophilins must be flexible enough to accommodate the large variety of client proteins in the cell and perhaps also recognize strategic hydrophobic residues that would otherwise promote aggregation. Further studies are clearly needed to resolve the relative contributions of the PPIase versus chaperone activities of the immunophilins *in vivo*. Until then it is prudent to assume that both activities contribute to assist large Pro-containing proteins to fold properly [34, 35, 40, 46, 47].

Physiological role of immunophilins

In a landmark study, Dolinski et al. [52] deleted all twelve immunophilins in *S. cerevisiae* individually and together and did not find any defect in a number of physiological parameters tested, at least under laboratory growth conditions. These included viability at various temperatures, arrest and recovery from α-factor pheromones or glycogen, mating properties and sporulation. Although this result may be taken to mean that immunophilins are non-essential, it is more likely that they have a requirement in specific environmental signaling cues or under natural stress. For example, in *E. coli*, all four periplasmic PPIases, viz. FkpA, PpiA, PpiD and SurA, are dispensable for growth, but SurA is required for biogenesis of the pilus, an organ essential for the ability of the bacteria to invade the urinary tract [53]. In *Bacillus subtilis*, PpiB (a cyclophilin) and TF (see below) are the only two cytoplasmic PPIases. Disruptions of both genes together has no effect on cell viability in rich medium or under several stress conditions, such as heat, osmotic or oxidative stress [54]. However, in poor medium, such as in the absence of amino acids, the growth of the double-mutant strain is strongly decelerated, indicating that the PPIases are essential for growth under starvation conditions. Thus, these PPIases appear to be non-essential for growth under pampering laboratory conditions but have significant roles in survival in environmental and pathogenic niches. There is now an enormous body of evidence suggesting that immunophilins play an essential role in various biochemical pathways, a few examples of which are provided below.

A variety of protein-folding processes depends on the PPIase and/or chaperone-like activities of the immunophilins. CypA is important for the maturation of oligomeric receptors [55] and the activity of essential Zn-finger proteins, YY1 and Zpr1 [56]. It is also linked to atherosclerosis, rheumatoid arthritis and endothelial dysfunction, and is produced by macrophages following lipopolysaccharide stimulation, suggesting its role as an inflammatory mediator [57–59]. Recent studies of knockout mice have clearly established a physiological role for CypA *in vivo* [58]. The CypA knockout mice are viable but spontaneously develop an allergic disease with elevated IgE and tissue infiltration by mast cells and eosinophils, reminiscent of interleukin (IL)-4 overexpression [60]. A search of its mechanism led to the discovery that the PPIase active site of CypA binds to a regulatory Pro residue of ITK, and thereby inhibits the latter. ITK is a Tec family tyrosine kinase crucial for the T helper 2 (Th2) response, and thus, this interaction suppresses the Th2 response in wild-type animals.

Interaction of the ER-resident CypB with prolactin potentiates nuclear retrotransport of prolactin, cell growth and proliferation. These effects are abrogated by the loss of the PPIase activity of CypB [61]. Genetic studies [62, 63] have identified a critical role for CypD, a component of the mitochnodrial membrane permeability transition pore, in apoptosis induced by calcium and reactive oxygen species (ROS) and in cardiac ischemia-perfusion injury. Cells from CypD-knockout mice were resistant to death imposed by excess calcium and ROS, but not death from X-ray, staurosporine, tumor necrosis factor, or expression of pro-apoptotic proteins [64]. These results also highlight CypD as a potential therapeutic target in tissue death and myocardial infarction.

CD147 is a widely expressed plasma membrane protein that has been implicated in a multitude of physiological and pathological activities, including the induction of extracellular matrix metalloproteinase, regulation of lymphocyte responsiveness and spermatogenesis [65]. Extracellular CypA and CypB act as potent lymphotactic agents by virtue of their interaction with CD147 on the membrane of these immune cells [66]. Agents targeting either CD147 or Cyps show a significant anti-inflammatory effect in animal models of acute or chronic lung diseases, suggesting a therapeutic avenue. CD147 also serves as a receptor for virus-associated CypA [67].

Interestingly, cell surface expression of CD147 itself requires another Cyp, namely Cyp60 [68].

Without doubt, our most detailed knowledge of immunophilin function derives from steroid receptors that depend on an ordered assembly of chaperoning proteins to reach functionally mature conformations [69, 70]. The constituents of these receptor complexes include the major cellular chaperone, heat shock protein 90 (Hsp90), the co-chaperones, Cyp40, FKBP51 and FKBP52 and the unique TPR-containing Ser/Thr phosphatase, PP5 [1, 32, 69–71]. In fact, all three immunophilins mentioned above were first identified in steroid receptor complexes. All have TPR domains that interact with the C-terminal peptide MEEVD of Hsp90 in their respective complexes [27]. Specificity of composition is an important feature of steroid receptor-immunophilin complexes. As a rule, FKBP51 is more abundant in the glucocorticoid receptor (GR) complexes, while Cyp40 is most abundant in complexes of the progesterone receptor (PR). As mentioned before, the C terminus of Hsp90 binds the TPR domains of the immunophilins, while a central region of Hsp90 likely interacts with the ligand-binding domain of the client receptor. This in turn results in the recognition of the receptor by the co-chaperoning immunophilins, likely via the PPIase domains of the latter. Thus, the specificity of recognition between an immunophilin and its cognate receptor can give rise to complexes of different steroid receptors, all of which will contain the primary chaperone, Hsp90 [71]. Recently, interaction between ryanodine receptor and FKBP12.6 (calstabin2) was shown to be important to rescue heart failure in a mouse model [72].

The other important property of the steroid receptor-immunophilin complexes is the variability of their composition in response to physiological stimuli. This is best known for steroid receptor signaling whereby FKBP51 and FKBP52 play an opposing role. FKBP51 has an inhibitory effect on GR and PR activities, and both the FKBDs and the Hsp90-binding TPR domain are important for this inhibitory function [73, 74]. FKBP52, on the other hand, opposes this inhibitory effect of FKBP51. In a recent discovery, binding of the glucocorticoid hormone to the GR was shown to stimulate a rapid replacement of FKBP51 by FKBP52 in the complex [75]. Following this exchange, FKBP52 promoted translocation of the receptor to the nucleus through its interaction with the motor protein, dynein, thus leading to the transcriptional activation of GR-responsive nuclear genes [75, 76]. Clearly, the dynamic nature of a steroid receptor complex lends itself to myriad regulatory scenarios. An interesting situation is presented in the New World primates, such as squirrel monkeys, that contain naturally high levels of FKBP51 and low levels of FKBP52. Recent studies have suggested that androgen insensitivity in these animals is determined primarily by the low levels of FKBP52 and not the high levels of FKBP51 [77].

While a chemical inhibitor such as FK506 (or CsA) may globally inhibit all FKBP (or Cyp) paralogs in the mammalian cell, a future goal is the rational design of specific drugs against individual immunophilins to affect a subset of complexes, thus allowing pharmacological intervention of specific signaling pathways. Knockout studies in mice have revealed specific non-redundant roles of the large FKBPs. Mice lacking FKBP52 grow normally but both sexes are sterile and exhibit multiple anatomical and functional abnormalities of the reproductive organs [78–80]. The female mice show complete failure of uterine implantation, a process that requires estrogen (ER) and two progesterone receptors, PR-A and PR-B. Loss of FKBP52 leads to a specific disruption of PR-A signaling but does not affect ER or PR-B function [80]. In male mice, loss of FKBP52 leads to altered urethral epithelial fusion causing ectopic openings of the penis, which is equivalent to hypospadias in the human, a common birth defect affecting approximately 1 in 125 males at birth [E. Sanchez, personal communication]. These results unravel a potentially novel mechanism in which FKBP52 regulates androgen receptor-mediated genital development in a tissue-specific manner. While the detailed mechanism of the various phenotypes of the FKBP52-null mice clearly needs further research, it should be appreciated that FKBP52 binds many partners beside the steroid receptors; they include interferon regulatory factor 4, and a novel FKBP-associated protein, FAP48, needed for increased IL-2 production [81], suggesting multiple and complex pathways. In plants, loss of multi-domain immunophilins, often located in the thylakoid lumen, generates strong developmental phenotypes [82].

Recent studies have documented critical roles of immunophilins in nerve regeneration [83, 84]. FKBP12 and FKBP52 levels are high in neurons, and are further elevated in facial or sciatic nerve injuries. Increased FKBP12 levels are found in areas of pathology in neurodegenerative diseases. FK506 promotes nerve regeneration in a PP2B-independent mechanism, and it has been proposed that this is due to the disruption of the steroid receptor complexes in the cell, which leads to the release of chaperones such as FKBP52, making them available for neurotrophic signaling pathways [84]. Clearly, it will be interesting to study the extent and nature of nerve regeneration in the available FKBP52 knockout mice. The results may provide important therapeutic avenues to treat a variety of neurodegenerative conditions that currently affect the aging human population, such as familial amyotrophic lateral sclerosis, Alzheimer's and Parkinson's diseases.

As a rule, the immunophilins are either constitutively active PPIases or enzymatically dead. Recently, however, an instance of regulation of PPIase activity has been reported [85]. Human FKBP38, consisting of a PPIase homology domain followed by TPR (similar to PfFKBP35 in Fig. 1)

is inactive in neuronal cells, but is activated when the intracellular Ca^{2+} concentration rises and the FKBP38/Ca^{2+}/calmodulin complex assembles. The newly formed PPIase site in the complex recruits and inhibits the anti-apoptotic protein Bcl-2, promoting neuronal apoptosis. Thus, FKBP38 PPIase is tightly regulated by signaling via a second messenger, although the precise role of this PPIase activity still needs to be resolved.

The list of human syndromes associated with the immunophilin defect seems to be growing, although the exact molecular underpinnings remain unknown. A chromosomal deletion at 7q11.2 is found in 95% of patients with Williams-Beuren syndrome, characterized by typical facial features, mental retardation, and aortic stenosis. The lost genes include elastin, LIM kinase-1, RFC2 and a gene named FKBP6 that codes for a 36-kDa immunophilin, FKBP36 [86, 87]. Polymorphism in FKBP51, which plays a role in the stress hormone-regulating hypothalamic-pituitary-adrenal axis, has been found to be related to a faster response to anti-depressant drug treatment and to increased recurrence of depressive episodes [88].

Finally, a few recent reports have drawn attention to the ability of immunophilins to inhibit PP2B without the need for immunosuppressive drugs. FKBP51 overexpression in megakaryocytes inhibits calcineurin activity, suggesting that FKBP51 physiologically regulates calcineurin activity either alone or by complexing an unknown cellular protein [89], which may underlie the pathogenesis of human idiopathic myelofibrosis (IMF) [90]. The genome of the parasitic protozoa, *Plasmodium falciparum*, the causative agent of malaria, contains a single FKBP homolog (PfFKBP35) whose predicted sequence contains a PPIase domain followed by three TPR motifs (Fig. 1) [30, 31]. The recombinant protein causes about 70% inhibition of the parasitic PP2B *in vitro* under optimal conditions, and addition of FK506 does not increase the inhibition. This is not due to the inability of PfFKBP35 to interact with FK506, because the PPIase activity of the protein is FK506 sensitive. It is clear that this novel FKBP intrinsically attains a structure that binds to PP2B and inhibits the latter, raising the question whether it serves as a physiological regulator of PP2B. Interestingly, in the *P. falciparum* cell, FKBP35 exhibits a stage-specific nucleocytoplasmic shuttling such that most of it does not co-localize with parasitic PP2B. Clearly, the physiological implication of the innate PP2B-inhibitory activity of immunophilins is an important new area that deserves to be explored.

Use of host immunophilins by parasites

Obligatory parasites co-opt a variety of host cell proteins for infection and growth, and immunophilins have been recently added to their menu. A large variety of Cyps and FKBPs are found associated with various viral macromolecules and/or packaged inside mature virions, although their role in viral growth is often unclear [91–95]. The HIV-1 Gag protein binds to a number of Cyps, although CypA may play a more specific role as it is packaged in HIV virions as well [37, 38, 92, 95–97]. A small region of the HIV Gag containing four conserved prolines has been shown to be important for incorporation of CypA into virions [94–96]. It is estimated that roughly one molecule of CypA is packaged per 10 Gags, which translates into about 250 CypA molecules per HIV virion [91]. CypB is not packaged in HIV-1. However, an interaction of CypB with hepatitis C virus (HCV) RNA-dependent RNA polymerase, NS5, was shown to be critical for NS5 function and viral genome replication [97]. Interestingly, HCV replication takes place in close association with the ER, where CypB also resides, offering an example of how a virus has evolved to use an immunophilin available in the organelle in which it grows. For both HIV and HCV, Cyp inhibitors also inhibit viral replication, providing an antiviral approach. CypA is also found associated with vesicular stomatitis virus, a negative stranded RNA virus, where it likely acts as a chaperone for the extremely hydrophobic nucleocapsid (N) protein that wraps the genomic RNA to produce the functional template for transcription [87]. Paralogs of N protein in other negative strand RNA viruses also use the viral phosphoprotein (P) as a chaperone and, thus, the relative importance of P and CypA in chaperoning N protein needs to be assessed. HIV-1 packages an average of 25 molecules of FKBP12 per virion [91]. FKBP12 seems to have a consensus client sequence of Phe-Pro, which is also an HIV-1 protease-specific cleavage site; nonetheless, the relevance of FKBP12 in HIV-1 has not been evaluated.

Future directions

There is no doubt that immunophilins play important roles in regulating the higher-order structure of proteins and in promoting protein-protein interactions. In view of the multiple immunophilins in practically every organism, a challenge would be to unravel the specific role of each paralog. Identification of interacting partners and the composition of distinct macromolecular complexes containing each immunophilin will be critical in this endeavor. An *in vivo* analysis will involve the determination of the phenotype of classical knockout mutants. For immunophilins that are essential for growth and viability, the preferred technique could be knockdown of expression through RNA interference, as was done for *T. gondii* FCBP [5]. One can then investigate the detailed biochemical mechanism of the phenotype including the possible disruption of the cognate complexes. Pharma-

ceutical inhibition of a specific immunophilin may be an unrealistic goal due to the considerable sequence similarity among the paralogs and the overall conservation of secondary structure. A more reasonable approach may entail the use of small molecular or peptidomimetic inhibitors targeting the specific domain of a client protein that interacts with the cognate immunophilin, thus abrogating a specific signaling pathway. The molecular and thermodynamic mechanism of how immunophilins promote protein folding will continue to be another area of active research. Determination of higher-order structure of complexes of immunophilins with various peptides and the use of biophysical techniques for fast kinetic resolution of the folding events should reveal important details of the pathway. Recent studies have shown that the positioning of the substrate in the active cleft activates an unknown number of remote 'subsites' in the transition state of the reaction [98]. The exact identity and number of such sites appear to vary between the PPIase families and must be experimentally determined. Three-dimensional structures of PfFKBP35 and its complex with PP2B also need to be determined to understand its innate PP2B-inhibtiory activity.

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