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Review

Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts

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Abstract. Cyclosporine A therapy for prophylaxis against graft rejection revolutionized human organ transplantation. The immunosuppressant drugs cyclosporin A (CsA), FK506 and rapamycin block T-cell activation by interfering with the signal transduction pathway. The target proteins for CsA and FK506 were found to be cyclophilins and FK506-binding proteins, (FKBPs), respectively. They are unrelated in primary sequence, although both are peptidyl-prolyl cis-trans isomerases catalyzing the interconversion of peptidyl-prolyl imide bonds in peptide and protein substrates. However, the prolyl isomerase activity of these proteins is not essential for their immunosuppressive effects. Instead, the specific surfaces of the cyclophilin-CsA and FKBP-FK506 complexes mediate the immunosuppressive action. Moreover, the natural cellular functions of all but a few remain elusive. In some cases it could be demonstrated that prolyl isomerization is the rate-limiting step in protein folding in vitro, but many knockout mutants of single and multiple prolyl isomerases were viable with no detectable phenotype. Even though a direct requirement for in vivo protein folding could not be demonstrated, some important natural substrates of the prolyl isomerases are now known, and they demonstrate the great variety of prolyl isomerization functions in the living cell: (i) A human cyclophilin binds to the Gag polyprotein of the human immunodeficiency virus-1 (HIV-1) virion and was found to be essential for infection with HIV to occur, probably by removal of the virion coat. (ii) Together with heat shock protein (HSP) 90, a member of the chaperone family, high molecular weight cyclophilins and FKBPs bind and activate steroid receptors. This example also demonstrates that prolyl isomerases act together with other folding enzymes, for example the chaperones, and protein disulfide isomerases. (iii) An FKBP was found to act as a modulator of an intracellular calcium release channel. (iv) Along with the cyclophilins and FKBPs, a third class of prolyl isomerases exist, the parvulins. The human parvulin homologue Pin1 is a mitotic regulator essential for the G2/M transition of the eukaryotic cell cycle. These findings place proline isomerases at the intersection of protein folding, signal transduction, trafficking, assembly and cell cycle regulation.

Key words. Peptidyl-prolyl cis-trans isomerases; protein folding; cyclophilins; FKBPs; parvulins; immunosuppression.

Introduction

Protein folding was thought to be a spontaneous process by transforming the linear primary sequence information of the nascent polypeptide chain into a well-defined three-dimensional structure, suggesting that all the information needed for proper folding is located in the primary amino acid sequence [1]. Refolding experiments of denaturated proteins show that fold-

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ing events of globular single-domain polypeptides occur in second or even millisecond time scales.

However, one relevant conformational change occurring during protein folding events takes significantly more time. This is the cis-trans isomerization of peptidyl-prolyl bonds (fig. 1). Most, but not all, peptide bonds are connected in the trans conformation during biosynthesis at the ribosomes, and this conformation is also found in the native structure of most peptide bonds. However, in case of the peptidyl-prolyl bond in a peptide backbone, both the cis and trans conformations are accessible.

In refolding experiments it could be demonstrated that the cis/trans isomerization of peptidyl-prolyl bond was rate limiting [2]. Folding intermediates are highly sensitive to proteolytic degradation and aggregation, indicating that an enzyme-catalyzed acceleration of proline isomerization may exist. In 1984 the first peptidyl-prolyl cis-trans isomerase (prolyl isomerase; PPIase EC 5.1.2.8) was isolated from porcine kidney cortex by Fischer et al. [3]. At that time an intensive search for the intracellular receptor for the immunosuppressive drug cyclosporin A (CsA) was underway. CsA is an undecapeptide (fig. 2) produced by a variety of fungi imperfecti, notably *Tolypocladium inflatum* [4]. Beginning in the 1980s, CsA was used as an immunosuppressive, and is now widely employed for prophylaxis and treatment of allograft rejection following human organ and tissue transplants. In 1984 an 18-kDa protein from mammalian thymocytes was found to be the intracellular receptor protein for CsA, and this receptor protein was named cyclophilin. Subsequently further cyclophilins were characterized by sequence from calf thymus [5] and a human T-cell line [6]. In 1989 it was demonstrated that cyclophilins and peptidyl-prolyl cis-trans isomerases were species variants of the same protein [7, 8]. At the same time, however, another prolyl isomerase was discovered [9, 10] during the search for the receptor protein of the immunosuppressive-acting drug FK506. FK506 is a polyketide (fig. 2) produced by *Streptomyces tsukubaesis* [11], a species discovered in a soil sample

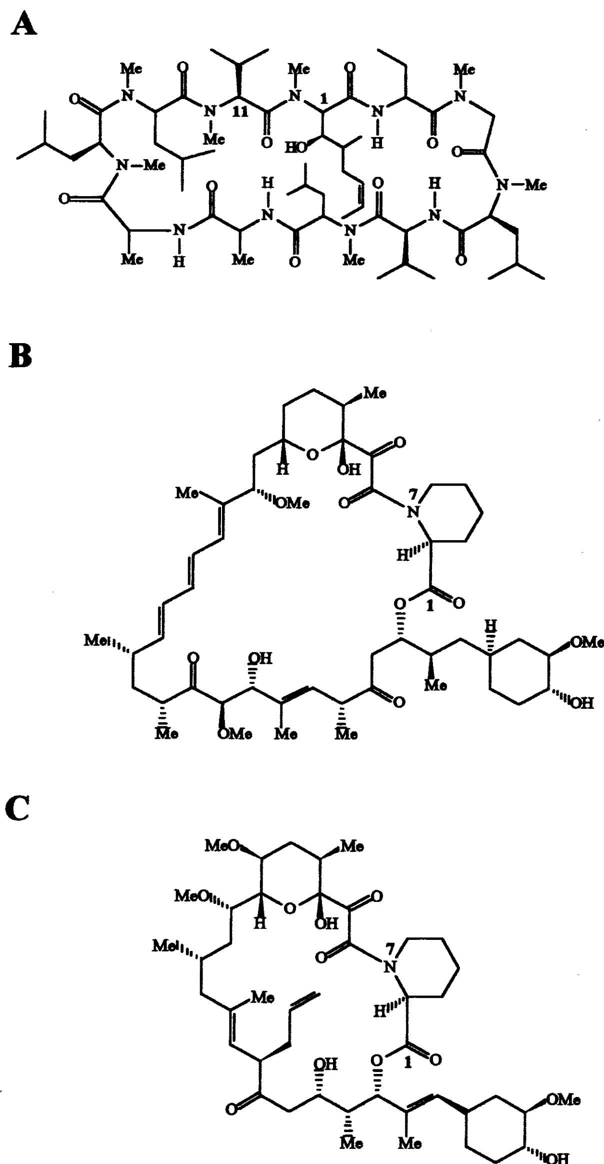

Figure 2. Structure of the immunosuppressive acting drugs CsA, rapamycin and FK506. (*A*) CsA is produced by a variety of fungi, notably *Tolypocladium inflatum*. (*B*) The antifungal agent rapamycin was isolated from *Streptomyces hygroscopius* (*C*) and the structurally related polyketide FK506 is produced by *Streptomyces tsukubaesis*.

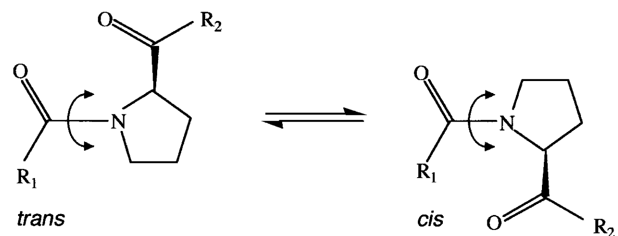

Figure 1. Cis-trans isomerization of a peptidyl-prolyl bond.

from Tsukuba, Japan. It is structurally related to rapamycin (fig. 2), which was first described in 1975 as an antifungal agent [12]. It was isolated from *S. hygroscopicus* found on Easter Island, and the name rapamycin comes from Rapa Nui, the native name for Easter Island. Using an FK506 affinity matrix, the receptor protein was isolated and named FK506-binding protein (FKBP). However, the amino acid sequence

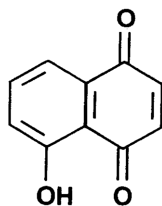

Figure 3. Structure of juglone (5-hydroxy-1,4-naphthoquinone). Juglone specifically inhibits enzymes of the parvulin family. In contrast to CsA and FK506, inhibition by juglone is irreversible.

of the FKBPs was found to be totally different from the cyclophilins, even in the active site [9, 10, 13]. It became obvious that the prolyl isomerases were divided into different classes. Using the binding criterion, prolyl isomerases, also called immunophilins, are divided into three families of unrelated amino acid sequence. Cyclophilins selectively bind CsA, whilst FKBPs selectively bind the drugs FK506 and rapamycin. For a long time no inhibitor was known for the third group [14, 15], the parvulins (see below), whose first member was isolated from *Escherichia coli* [16]. Recently, it was demonstrated that juglone, 5-hydroxy-1,4-naphthoquinone (fig. 3), irreversibly inhibits the enzymatic activity of several parvulins [17]. The scope of this review will be limited to the most recent aspects of the structure-function relationship of the three major prolyl isomerase families in pro- and eukaryotes and to various aspects of their peptidyl prolyl cis-trans isomerase activities in vitro and in vivo.

The cyclophilin family

Cyclophilins are ubiquitous proteins highly conserved during evolution. They are found in bacteria, fungi, plants and vertebrates, and are widely expressed in many tissues. At least eight different forms of human cyclophilins have been found, ranging from 18 kDa to 150 kDa in molecular mass (table 1). Cyclophilin A (CyPA) is a cytosolic protein with a molecular mass of 17.7 kDa. CyPA binds CsA in nanomolar concentrations (IC_{50} 6 nM [18]) and appears abundantly expressed in all tissues. The fact that CyPA is not T-cell specifically expressed is thought to be one factor causing side effects in clinical CsA therapy. T cells contain about 3–7 μg of CyPA per milligram of total protein [19]. A large content of CyPA was found in splenic erythrocytes and myeloblasts [20]. CyPA was found in different cells of the rat brain, relatively high levels occurring in the cerebral cortex and hippocampus. This level is higher in the neurons than in the glial cells [21].

Several other mammalian cyclophilin isoforms have been cloned and characterized biochemically. All share sequence homology with CyPA of over 50%. Cyclophilin isoforms differ in their subcellular localization and binding affinity to CsA. Many of them contain an N-terminal leader sequence delegating the cyclophilin to their specific subcellular locations. CyPB is a 21-kDa protein containing an N-terminal signal sequence thought to mediate translocation into the endoplasmatic reticulum (ER) [22]. Immunofluorescent staining revealed that the avian homologue of CyPB, s-cyclophilin, is colocalized with the calcium storage protein calreticulin, suggesting that CyPB could play a role in Ca^{2+}-dependent signalling pathways [23]. Like CyPB, the CyPC protein is localized in the ER. It is 23 kDa in size, contains an N-terminal signal sequence and was isolated from a murine T-cell line [24]. CyPC shows a higher expression rate in kidney compared with liver and T cells. In the absence of CsA, CyPC fusion proteins bind specifically to a 77-kDa protein. Although the function of the 77-kDa protein is unknown, it was found to possess a domain common to many cell sur-

Table 1. The yeast cyclophilins.

Gene	Molecular mass	Mammalian homolog	Localization	Characteristics
CPR1	17 kDa	CypA	cytoplasm	primary receptor for CsA
CPR2	20 kDa	CypB	secretory pathway	induced by heat shock and tunicamycin
CPR3	20 kDa	CypD	mitochondria	deletion has growth on lactate at 37 °C accelerates Su9-DHFR refolding
CPR4	33 kDa	CypC	secretory pathway	induced by heat shock and tunicamycin
CPR5	23 kDa		ER	induced by tunicamycin
CPR6	45 kDa	Cyp40	cytoplasm	binds together with Hsp90 to inactive steroid receptor
CPR7	45 kDa	Cyp40	cytoplasm	binds together with Hsp90 to inactive steroid receptor
CPR8	35 kDa	CypC	secretory pathway	*CPR4* homolog

face proteins, including the scavenger receptor. In the presence of CsA, CyPC binds specifically to a 55-kDa protein. The 55-kDa species was shown to be a Ca^{2+}- and calmodulin-dependent serine/threonine phosphatase called calcineurin [25].

CyPD (18 kDa) contains a mitochondrial signal sequence, and the cyclophilin NK-TR (150 kDa) is thought to be a part of the tumour recognition complex on the surface of natural killer cells. The CyP40 proteins contain an N-terminal domain with significant sequence homologies to the CyP18. CyP40 forms a chaperone-like complex with the heat shock protein HSP90 required for proper protein folding of steroid receptors [26–28].

Recently a novel spliceosomal-specific cyclophilin, SnuCyp-20, was identified [29]. The spliceosome catalyzing the removal of introns from nuclear pre-messenger RNA (mRNA) molecules is a complex consisting of the small nuclear ribonucleoprotein (snRNP) particles U1, U2, U4/U6 and U5, as well as an undefined number of non-snRNP splicing factors. The 20-kDa SnuCyp-20 of human [U4/U6.U5] tri-snRNPs forms a complex with the U4/U6-specific 60-kDa and 90-kDa proteins [29]. The role and function of the novel cyclophilin during the splicing event is under investigation (R. Lührmann, personal communication).

Today the genomic DNA sequences of *Haemophilus influenzae*, *Mycoplasma genitalium*, *Bacillus subtilis* and *Saccaromyces cerevisiae* are known, revealing the complete inventory of four living organisms. It is thought that *M. genitalium* contains the smallest genome of a free living organism, thereby representing the near-minimal set of genes necessary for life. For the cyclophilins, a drastic multiplication in higher organisms did occur. Although yeast has seven cyclophilins, only two were found in *E. coli*, one in the cytosol and one in the periplasm [30]. *H. influenzae* and *B. subtilis* possess only one cytosolic cyclophilin each [30, 31]. In *M. genitalium*, no homologue was found. Like the human prolyl isomerases (PPIases), the yeast cyclophilins are localized in different compartments. Cpr1 is homologuos to the human CyPA, found in the cytosol. Cpr2 is located in the ER. Mitochondrial Cpr3 was shown to be required for acceleration of protein refolding after mitochondrial import and at elevated temperatures. Cpr6 and Cpr7 are homologues of the CyP40 class of proteins. Both bind to HSP90 and are required for proper steroid hormone folding. Cpr4 and 5 are both located in the ER. The precise function is not yet known [30].

Structure of human CyPA in complex with CsA and peptide substrate

Structure analysis of human CyPA [32] (also called hCyP18) and the periplasmatic eCyP of *E. coli* [33, 34] demonstrated that the cyclophilins are structurally highly conserved. Cyclophilins are folded as a β-barrel with eight β-strands connected by loops and three α-helices. The hydrophobic active site of the human cyclophilin CyPA is formed by the residues R55, I57, Q63, A102, Q111, F113, L122, H126 and R148 [35]. The cis-proline binding pocket of human CyPA is formed by the amino acids F60, M61, F113 and L122. For these investigations the model tetrapeptide acetyl-Ala-Ala-Phe-Pro-amidomethylcoumarin was used.

X-ray structure analysis of human cyclophilin-CsA complex showed that CsA was bound in a very similar fashion in the active site as the model tetrapeptide. The structure of bound CsA is substantially altered when compared with unbound CsA. In the bound state, the side chains are flipped over from the inside to the outside. Thirteen contact residues with CyPA were found: R55, F60, M61, Q63, A101, N102, Q111, F113, L122, H126 and R148. Hydrogen bonds were found between MeBmt-1 γOH (CsA) and N102 CO (CyP), MeBmt-1 CO Gln-63 εNH, MeLeu-9 CO and W121 εNH, MeLeu10CO and Arg55 ηNH, and between MeVal11CO and His126 εNH [36]. The importance of some of these residues in CsA binding was underlined by site-directed mutagenesis experiments. Exchange of the W121 to phenylalanine or alanine led to a 200- and 400-fold reduced CsA sensitivity of the mutant proteins, respectively [37].

The precise knowledge of the structure of the cyclophilins and the structure-function relationships with the different substrates and inhibitors, as well as the fact that cyclophilins contain a hydrophobic deep binding pocket, were used to engineer a cyclophilin into a proline-specific endopeptidase [38]. Within the proline binding site, a catalytic triad (Ser-His-Asp) known from peptidases was introduced. Already a single exchange from alanine to serine (A91S) showed a specific protease activity in the *E. coli* cyclophilin. With the help of site-directed mutagenesis experiments the complete catalytical triad was introduced into the eCyP. The catalytic efficiency k_{cat}/K_M for proteolytic cleavage was more than 108-fold enhanced compared with the wild type [38].

Function of cyclophilin A during HIV-1 infection

The viral Gag protein and its proteolytically derived maturation products serve as the major structural components of the human immunodeficiency virus-1 (HIV-1) virion. Late in the infectious cycle, Gag accumulates at the cell membrane and assembles into immature virions which bud from the cell. Gag plays the central role in this assembly process and can form budded, viruslike particles in the absence of any other viral

Table 2. The yeast FKBPs and further mammalian homologues.

Gene	Molecular mass	Mammalian homologue	Localization	Characteristics
FPR1	12 kDa	FKBP12	cytoplasm	primary receptor for FK506 and rapamycin
FPR2	12.5 kDa	FKBP13	secretory pathway	induced by hear shock and tunicamycin
FPR3	70 kDa	FKBP25	nucleolus	phosphorylated
FPR4	60 kDa	FKBP25	nucleus	FPR3 homologue
-	12.6 kDa	FKBP12.6	cytoplasm	associated with the cardiac calcium release channel
-	33 kDa	FKBP33	cell membrane	contains two FKBP binding domains
-	51 kDa	FKBP51	cytoplasm	only FKBP expressed primarily in T cells
-	52 kDa	FKBP52	cytoplasm	binding toHSP90 in the inactive steroid hormone receptor

protein. As the immature virion buds, the membrane-bound Gag protein is cleaved by the viral protease into three new proteins: matrix (MA), capsid (CA) and nucleocapsid (NC), as well as three smaller polypeptides. The CA protein forms a distinctive conical core structure which encloses the NC/RNA complex of the virion. The viral particle must subsequently disassemble or rearrange when the virus infects a new cell in order to allow reverse transcription of the RNA genome and active transport into the nucleus [39].

CyPA has been reported to bind human HIV-1 Gag protein and is required for the infectious activity of HIV-1 virions [40, 41]. HIV replication requires packaging of the cellular cyclophilin A. CyPA binds directly to the CA domain of Gag, and the formation of the CA/CyPA complex is competitively inhibited by CsA. The precise function of CPA during infection is not yet known. However, the crystal structure of CyPA complexed with a 25-amino acid peptide of HIV-1 Gag capsid protein showed, that the sequence Ala88-Gly89-Pro90-Ile91 of the Gag fragment is the major portion bound to the active site of CyPA [42]. Interestingly, the peptidyl-prolyl bond between Gly89 and Pro90 of the fragment has the trans conformation, in contrast to the cis conformation observed in other known CyPA-peptide complexes (see above). Gly89 preceding proline has an unfavourable backbone formation usually only adopted by glycine, and it was suggested that this special Gly-Pro sequence is required for the binding of HIV-1 Gag protein to CyPA. Therefore CyPA was thought to act as a molecular chaperone, probably in the coating/uncoating processes during the infectious life cycle rather than acting as a cis-trans isomerase.

The FKBP family

Members of the FKBP family ranging in size from 12 to 52 kDa were found in all organisms investigated (table 2). FKBP12 (a 12-kDa protein) is an abundant, ubiquitously expressed cyctosolic protein possessing prolyl isomerase activity which is inhibited by the makrolides FK506 and rapamycin in nonmolar concentrations. FKBP12 comprises 108 amino acids which are highly conserved between human and rabbit (100%) as well as human and rat (95%). Human FKBP12 binds FK506 with a K_D of 0.4 nM or rapamycin with a K_D of 0.2 nM [43].

A cellular function for one FKBP12 member of this immunophilin family as an integral component of the intracellular calcium release channel complex has been recently established (for review see [44]). Two prototypic members of this calcium release family, the skeletal muscle ryanodine receptor (RyR1) and the inositol 1,4,5-triphosphate (IP_3) receptors, form tetrameric channels. This RyR1/calcium release channel comprises four ryanodine receptor proteins each with a molecular weight of 565,000 Da, giving a mass of nearly 2.3×10^6 Da for the functional channel. The stoichiometry of the association is such that one molecule of FKBP12 is associated with each RyR1 molecule (thus there are four molecules of FKBP12 per channel complex). As part of the channel complex, FKBP12 exerts profound functional effects on the channel gating. Closely related to FKBP12 is FKBP12.6 with 85% amino acid sequence identity to FKBP12. This FKBP12 orthologue, FKBP12.6, was believed to bind selectively to the cardiac ryanodine receptor RyR2 [45, 46]. To define a possible function for FKBP12 in vivo, a mutant mouse deficient in FKBP12 was recently generated [47]. The absence of FKBP12 was found to alter the single-channel properties of both cardiac RyR2 and skeletal RyR1, resulting in severe dilated cardiomyopathy [47].

FKBP13 is localized in the ER and has 43% homology to FKBP12. Yeast FKBP13 has an N-terminal hydrophobic signal sequence, whereas at its C-terminal moiety the ER-retention signal sequence was found. The 25-kDa rapamycin-specific FKBP25 protein was purified to homogeneity from brain, spleen and thymus [48]. Like other high molecular mass FKBPs, FKBP25 shares significant sequence similarities with FKBP12.

However, whereas FK506 and rapamycin bind and inhibit equally well the prolyl isomerase activity of FKBP12, bovine FKBP25 showed more than 200-fold preference for rapamycin over FK506. *Legionella pneumophilia* contains a basic FKBP25 (pI = 9.8) located on the bacterial cell surface [49]. It is thought that FKBP25 is involved in macrophage infectivity (the protein was called macrophage infectivity potentiator, MiP). Genetic experiments have proved, however, that MiP does not assist in the entry of *Legionella* to macrophages but rather helps the pathogen to survive inside the cell [50].

In *Streptomyces chrysomallus* the FKBP33 and FKBP12 proteins are encoded from a single operon. The nucleotide sequence of the region 5′ to the *fkbA* gene, encoding *S. chrysomallus* FKBP12, revealed an open reading frame (*fkbB*) encoding the FKBP33 protein [51]. Both genes are transcriptionally coupled under the control of a promoter localized upstream of *fkbB*. FKBP33 contains two FK506 binding domains with 43% and 32% identity to FKBP12, and both the individual domains as well as FKBP33 itself possess prolyl isomerase activity. FKBP33 is located in the cell membrane of *S. chrysomallus* and of other streptomycetes.

From a murine complementary DNA (cDNA) fragment, human FKBP52 was cloned and expressed in *E. coli*. Like the CyP40 proteins, FKBP52 is associated with HSP90 proteins in untransformed mammalian steroid receptor complexes [52]. However, the HSP90 binding site was found to be distinct from the prolyl isomerization binding site. FKBP52 and CyP40 contain a three-unit tetratricopeptide repeat. FKBP52 contains three FKBP12-like domains. Domain 1 shows all the structural features required for prolyl isomerization activity and FK506 binding. Domain 2 contains ATP and GTP binding sites. Domain 3, also structurally related to the FKBPs, is followed by a C-terminal domain that contains the calmodulin-binding consensus sequence. Thus FKBP52 may be involved in multiple immunological, endocrinological and chaperone-mediated pathways. Closely related to FKBP52 is FKBP51, cloned from murine T cells with 53% identity with murine FKBP52.

A data bank search revealed that yeast contains four FKBP proteins: fpr1 is a cytosolic FKBP, Fpr2 is located in the ER, Fpr3 is nuclear prolyl isomerase and Fpr4 is membrane-bound [30]. Using the yeast two-hybrid system, aspartokinase, an enzyme catalyzing an intermediate step in the threonine and methionine biosynthesis, was found to be an in vivo target of FKBP12. However, FKBP12 is not required for aspartokinase expression or activity. Instead, FKBP12 regulates feedback inhibition of aspartokinase, possibly by catalyzing conformational changes induced by product binding [53]. Four homologues of the FKBPs were found in *E. coli* and two in *H. influenzae* with specialized functions. For example, *E. coli* FkbA is a homologue of the pathogenicity factor from *L. pneumophilia* [54], and SlyD is a metal-binding protein [55].

Although no FKBPs are known from *B. subtilis* and *M. genitalium*, a homologue—the trigger factor—was found in all sequenced microorganisms but not in eukaryotes. The trigger factor is a three-domain protein with a molecular mass of 48 kDa. The internal domain has significant sequence similarities to the FKBPs [56], and therefore the trigger factor was classified among the FKBPs. However, the *E. coli* trigger factor was found to have no FK506 affinity at all, and therefore the trigger factor was put into a novel fourth class [57]. By now, many investigators share the idea that the trigger factor belongs to the FKBP family (as we contend in this review).

Cytosolic events controlled by CsA and FK506

The immunosuppressive actions exerted by CsA and FK506 on T cells and B cells have been discussed in numerous reviews [58, 59]. FK506 and CsA inhibit Ca^{2+}-dependent events which are primarily related to the suppression of transcription of early T-cell-activation gene products such as IL-2, IL-4 (interleukin 2 and 4), granulocyte-macrophage colony-stimulating factor (GM-CSF), and γ-interferon (fig. 4). One of the best-studied proteins in this spectrum of growth hormones is IL-2. Antigen recognition by T-cell receptors initiates signal transduction cascades resulting in rapid activation of the tyrosine kinases. This is followed by tyrosine phosphorylation of phospholipase C (PLC), generating the second messengers phosphatidylinositol bisphosphate (PIP_2) and IP_3, which causes the elevation of free intracellular Ca^{2+} and 1,2-diacylglycerol (DAG). Through the increased level of free Ca^{2+}, a complex of calmodulin and calcineurin is formed. Calcineurin, a calcium/calmodulin-dependent serine-threonine phosphatase, dephosphorylates the nuclear factor of activating T cells (NF-AT). In its dephosphoryted form NF-AT is able to cross the nuclear membrane to function as a transcription activator for IL2 expression (fig. 4).

Cyclophilins and FKBPs, together with their specific immunosuppressive-acting ligands CsA and FK506, respectively, are able to interfere with this signal transduction pathway. The FKBP-FK506, as well as the cylophilin-CsA complexes, bind to calcineurin and inhibit phosphatase activity. High-resolution X-ray structure of the FKBP-FK506 complex [60–62] shows that half of the FK506′s solvent-accessible surface area is buried in FKBP12; the other half of the ligand is exposed. Thus, FK506 has two domains: the binding

domain that interacts with FKBP12, and the effector domain that can interact with a second protein (e.g. calcineurin). It is the extended novel surface, build by the FKBP-FK506 complex but not the inhibition of the prolyl-isomerase activity, that causes the interference with the signal transduction pathway.

The parvulin family

In contrast to the cyclophilins and the FKBPs, only a limited number of parvulins or homologues are now known. The first parvulin (Par10 or PPiC) was originally isolated from *E. coli* [14, 15] and has a molecular mass of 10.1 kDa (the name 'parvulin' originates from the Latin word *parvus* = small). For a long time no inhibitor was known for the parvulin family, but recently juglone (5-hydroxy-1,4-naphtoquinone) was found to irreversibly inhibit the enzymatic activity of several parvulins [17]. Sur A is a homologue of Par10 in *E. coli* [63]. Sur A is located in the periplasm of *E. coli* and is necessary for survival during the stationary phase. Protein folding in the periplasm is thought to be different from folding in the cytosol [64]. The periplasm has wide pores in the outer membrane, and small molecules such as ATP are not found in the periplasm. Classical chaperones like GroEL/ES act in an ATP-dependent manner and are not found in the periplasm. Instead, protein folding in the periplasm is catalyzed by protein disulphide isomerases (PDIs) and prolyl isomerases. It was found that SurA assists in folding of OMPs (outer membrane proteins) [63] and can promote folding of many otherwise unstable proteins (reviewed in [65]). However, very little prolyl isomerase activity was found for the SurA protein [66]. Instead, SurA is thought to act as a periplasmatic chaperone. Previously, a novel parvulin homologue, PpiD, was identified in the periplasm of *E. coli*. The gene encoding PpiD was isolated as a multicopy suppressor of surA, a mutation which severely impairs the folding of OMPs. The protein is anchored to the inner membrane via a single transmembrane segment, its catalytic domain facing the periplasm [67].

In *B. subtilis*, the 33-kDa PrsA protein exists, bound to the outer face of the cytoplasmic membrane. As for the SurA protein, the prolyl isomerization activity is about 100- to 1000-fold lower compared with the *E. coli* Par10 (M. Sarvas, personal communication). PrsA is essential for protein secretion like α-amylases [68]. Further, parvulin homologues were also found in *H. influenzae* and other microorganisms [30].

Two essential parvulin homologues are known in eukaryotic cells: the human Pin1 and its budding yeast homologue Ess1, both acting as regulators in mitotic cell cycle [69]. Using a two-hybrid screen, Pin1 was

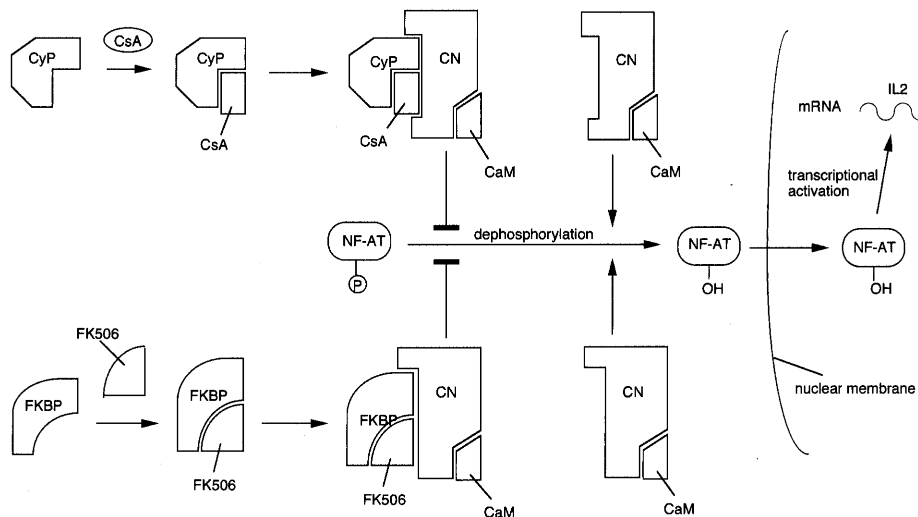

Figure 4. Hypothetical events associated with the CyPA-CsA and FKBP-FK506 complexes. During T-cell activation calmodulin (CaM) binds to the phosphatase calcineurin (CN) as a result of elevated levels of free intracellular calcium. This complex dephosphorylates the nuclear factor of activated T cells (NF-AT) which in the dephosphorylated form is able to cross the nuclear membrane. In the nucleus NF-AT acts as a transcriptional activator for interleukin-2 (IL-2) transcription. Cyclophilins (CyP) bind to cyclosporin A (CsA) and form a complex. The structure of bound CsA is substantially altered when compared with unbound CsA, and a novel surface is build by the CyP-CsA complex. The CyP-CsA complex binds to calcineurin and blocks the phosphatase activity of the CN/CaM complex. FKBP in complex with FK506 binds and inhibits the phosphatase activity of CN in an analogous way.

identified to interact with the NIMA kinase. Pin1 is a 163-amino acid polypeptide with an N-terminal putative protein-protein interaction domain [70], called WW domain, and a C-terminal prolyl isomerization domain. The eukaryotic cell cycle is characterized by defined periods of preparation for chromosome replication (G1), DNA replication (S), preparation for mitosis (G2), and mitosis (M). Depletion of Pin1 in HeLa cells or ESS1 in yeast resulted in mitotic arrest, whereas overexpression of Pin1 in HeLa cells caused G2 arrest.

Structure of the parvulins and functional analysis

Recently, the crystal structure of Pin1 complexed with the AlaPro dipeptide was solved [70]. The overall fold of the Pin1 WW domain was found to be similar to other WW domains. The C-terminal PPIase domain consists of four antiparallel β-sheets. In addition, four α-helices surround the flattened half β-barrel. A set of conserved residues in the Pin1/ESS1 class defines the binding pocket for proline (L122, M130, F134) and the peptide bond that undergoes cis-trans isomerization. In addition, a triad of basic side chains consisting of K63, R68 and R69 were found to sequester a crystallographically well-ordered sulfate ion, which led to a better understanding of the role of Pin1 during mitosis. The sulfate ion is close to the methyl group of the alanine in the bound AlaPro dipeptide, and therefore the sulfate ion was suggested to mimic a phosphorylated natural substrate [71].

The overall principle underlying cell cycle regulation is the appropriately timed structural modification of proteins through phosphorylation and dephosphorylation as well as specific protein degradation [72, 73]. For example, the G2/M transition in all eukaryotic cells requires CDC2, a serine/threonine kinase with catalytic specificity for phosphorylated peptidyl-prolyl groups. CDC2 activity requires the association of cyclin B because the activity of the cyclin B/CDC2 is negatively regulated by phosphorylation through the Wee1 and Myt1 protein kinases and positively regulated by the dephosphorylation of Thr14 and Tyr15 through CDC25C phosphatase. In order to test the idea that phosphorylated peptides might be the natural substrates of the Pin1 prolyl isomerase, phosphorylated serine (pS) substrate libraries of the general sequence H2N-MAXXXpSXXXAKK were investigated, and GST-Pin1 was found to bind preferentially to a subset of peptides with Pro immediately C-terminal to pS [71]. Whereas Pin1 exhibited little isomerase activity with substrates containing S/T-P bonds, phosphorylation of these peptides on Ser or Thr resulted in an up to a 300-fold increase in k_{cat}/K_M values.

These results suggest a model for mitotic regulation. Phosphorylation at specific S/T-P sites by mitotic kinases creates a binding site for Pin1 [71], which in turn induces conformational changes by catalyzing prolyl isomerization. Such local conformational changes could alter the activity of phosphorylated proteins such as NIMA kinase or Cdc25. Inactivation via partial unfolding of active sites has been shown for several enzymes, for example D-glyceraldehyde-3-phosphate dehydrogenase, ribonuclease A and creatine kinase. Given that inhibition of Pin1 induces mitotic arrest and apoptosis, this Pin1-mediated mechanism is a potential target for cancer therapy, and the novel discovery of the inhibitor juglone (fig. 3) is possibly the first step towards this task [17].

The cis/trans prolyl isomerase activity

Originally the prolyl isomerase activity was determined by an assay based on isomer-specific proteolysis using tetrapeptide derivatives. In proline-containing peptides about 10% of the peptidyl-prolyl bonds are in the unusual cis conformation. However, most proteases specifically cleave the all-trans conformer of a peptide substrate, and therefore proteases can be used to disturb cis-trans equilibrium of peptidyl-prolyl isomers (fig. 5). Common substrates therefore have the general structure Suc-Ala-Xaa-Pro-Phe-4-nitroanilide, with Xaa for any natural amino acid. In this case α-chymotrypsin is used as the helper protease. In a rapid reaction the chromogenic nitroanilide bond is cleaved to release the chromophore 4-nitoaniline (fig. 5). The slow cis to trans isomerization of peptidyl-prolyl bonds is monitored in the next step at 390 nm by the transient proteolysis [3].

For most part of the reported kinetic constants of PPIase catalysis have been evaluated with this type of peptide. This investigation demonstrated that the prolyl isomerases were perfectly adapted enzymes according to their catalytic function. In some cases rate constants k_{cat}/K_M close to the diffusion limit of 2×10^8 M^{-1} s^{-1} were reported [74]. The standard spectrophotometric assay for PPIases, which uses isomer-specific proteases as helper enzymes, is simple and requires a minimum of effort for sample preparation and experimental equipment. It can be used for screening and purification of novel prolyl isomerase out of crude extracts. However, only one direction of the reversible reaction (the cis to trans isomerization) can be followed. Due to the fact that only about 10% of the substrate (cis content) is monitored, the signal-to-noise ratio can cause problems as well as the solubility of the peptide substrate. The finding that the cis isomer content of the standard substrate can be shifted up to 70% in a trifluorethanol (TFE) solution containing 0.47 M LiCl by high solubil-

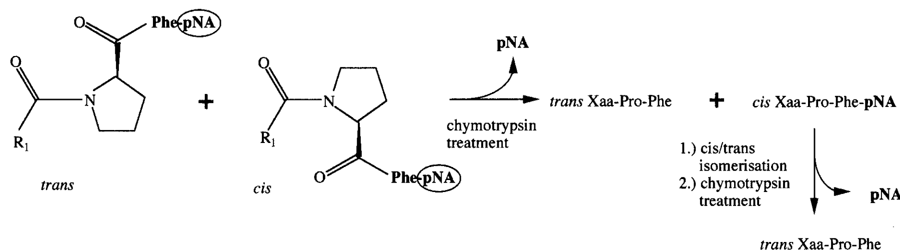

Figure 5. Mechanism of the assay to monitor peptidyl-prolyl cis-trans isomerization based on isomer-specific proteolysis. Common substrates have the general structure Suc-Ala-Xaa-Pro-Phe-pNA (Xaa is any amino acid; pNA, para-nitroanilide). α-Chymotrypsin is used as a helper protease. In a fast reaction the chromogenic nitroanilide bond is cleaved to release the chromophore 4-nitroaniline (pNA) of all-trans substrate molecules. The second step is the cis to trans isomerization of the peptidyl-prolyl bond of the tetrapeptide substrate. The following cleavage of the chromophore can be monitored at 390 nm.

ity minimized the signal-to-noise ratio as well as solubility problems in solvent jump experiments [75, 76]. This was important in determining the Michaelis constants and turnover numbers for proline isomerases. Constants of $K_M = 870$ μM (cis) and $k_{cat} = 12{,}700$ s^{-1} (cis) for human CyPA with the well-catalyzed substrate Suc-Ala-Ala-Pro-Phe-4-nitroanilide were determined in such solvent jump experiments [75]. With this data the catalytic efficiency of PPIases, defined as k_{cat}/k_u (k_u is the uncatalyzed prolyl isomerization), could be determined, and an acceleration of 5×10^6 for the cyclophilin-catalyzed prolyl isomerization at 0 °C was demonstrated, showing that prolyl isomerases work with very high efficiency [75].

The protease-coupled, irreversible assay still has several limitations, namely (i) the kinetic analysis is only possible under nonequilibrium conditions; (ii) only the cis to trans and not the reverse trans to cis isomerization can be investigated; and (iii) proteolytic products of the helper protease can influence the PPIase activity. These problems can be overcome by dynamic nuclear magnetic resonance (NMR) spectroscopy [77]. The uncatalyzed cis-trans isomerization is slow on NMR time scale, and therefore distinct signals occur for the trans and cis isomers in the one-dimensional ^{1}H NMR spectrum. Line-shape analysis of proton signals performed in the presence of various concentrations of porcine kidney CyP18 and peptide substrate revealed that catalysis of the cis-trans isomerization by CyP18 is best described by a four-site exchange model [77], where the four sites represent the cis and trans isomers free in solution and bound to the enzyme. For the substrate Suc-Ala-Phe-Pro-Phe-4-nitroanilide, the following kinetic constants were determined: $k_{cat} = 620$ s^{-1} and $K_M = 220$ μM for the trans isomer, and $k_{cat} = 680$ s^{-1} and $K_M = 80$ μM for the cis isomer, demonstating that CyP18 has a higher affinity for the cis isomer. The low k_{cat} value determined by NMR analysis compared with the high value in the protease-coupled assay originated in part from substrate specificity of the Xaa-Pro bond. However, dynamic ^{1}H NMR analysis is time-consuming in data analysis and requires large-scale technical equipment.

A novel, protease-free, spectrophotometric assay for the use of the standard peptide substrate is a welcome alternative. This assay is based on the small difference in absorbance determined for the two isomer forms. Molar absorption coefficients of 13,100 and 12,500 M^{-1} cm^{-1} were found for the cis and trans isomers of Suc-Ala-Xaa-Pro-Phe-4-nitroanilides, at 330 nm, respectively [78]. To increase the amplitude, substrates were dissolved in 0.47 M LiCl/TFE and the reaction started by solvent jump into aqueous buffer solution. The detected signal represented the sum of the spontaneous cis to trans and trans to cis prolyl isomerization. The difference of the observed constants obtained from the protease-free and the proteolytically coupled assay represents the rate constant for the trans to cis isomerization. Therefore, combination of protease coupled with uncoupled assays enabled Janowski et al. [78] to determine the complete set of the Michaelis-Menten parameters for prolyl isomerization in both directions. The only limiting factor is the unfavourable signal-to-noise ratio due to the small difference in absorbance of the two isomers.

The methods presented above are based on the analysis of small peptides. Instead, the putative in vivo function of prolyl isomerases includes prolyl isomerization in protein substrates for folding processes and signal transduction events. One step to close the gap between in vitro kinetics and in vivo function is the analysis of protein folding acceleration by addition of prolyl isomerases. Refolding kinetics of denatured proteins have

been shown to be an important tool for analyzing protein-folding mechanisms. Therefore, (i) the favourite protein substrate must contain a proline residue that is indeed cis-trans isomerized during protein folding, (ii) the cis-trans isomerization of a peptidyl-prolyl bond must the rate limiting step in protein folding, and (iii) unfolding and refolding have to follow a measurable time scale. The ribonuclease T1 (RNase T1) was shown to be such a favourable candidate. The three-dimensional structure of RNase T1 [79], solved by X-ray structure analysis, revealed that the enzyme contains four proline residues, two of which are in the cis conformation (*cis*-Pro39 and *cis*-pro55) and the other two being in the trans conformation (*trans*-Pro60 and *trans*-Pro73). Native RNase T1 is a rather stable protein due to two disulfide bonds connecting Cys2 with Cys10 and Cys6 with Cys103, respectively. During denaturation/refolding kinetics, this oxidized RNase T1 rapidly forms folded intermediates with reduced accessibility for proline residues. The reduced and carboxymethylated form (RCM RNase T1) is only marginally stable, and partially folded intermediates do not accumulate during folding. Proline residues therefore remain easily accessible for the catalysis of the trans to cis isomerization. Due to the two cis prolines other phases of slow kinetics occur during refolding as seen in fluorescence experiments [2]. However, replacement of one cis proline residue simplified the mechanism of RNase T1 folding, and the cis-trans isomerization of a single proline residue could be monitored [80].
Other protein substrates have been used to demonstrate that prolyl isomerases are able to accelerate protein folding [81]. However, we will focus on the studies on RNaseT1 because all three prolyl isomerase families have been studied in detail with this particular substrate.

PPIases as folding catalysts

All three prolyl isomerase classes—members of the cyclophilins from different species, human FKBP and *E. coli* parvulin—have been found to accelerate prolyl isomerization in protein substrates [82–85]. Using the reduced and carboxymethylated form of (S54G/P55N)-RNaseT1, k_{cat}/K_M of 73,000, 30,000 and 13,000 M^{-1} s^{-1} were observed for a CyPA, FKBP12 and the parvulin, respectively. A dramatically higher catalytic efficiency could be observed for the trigger factors from *E. coli*, *B. subtilis* and *M. genitalium* [31, 57, 85], all of which catalyze prolyl isomerization with reduced RNaseT1 about 20–100-fold (k_{cat}/K_M values in the range of $1–1.5 \times 10^6$ M^{-1} s^{-1}) better compared with the CyPs, FKBPs and parvulin.
Originally, the trigger factor was discovered by its ability to maintain the precursor protein proOmpA (outer membrane protein A) in a translocation-competent conformation [86, 87]. Subsequent studies, however, in which the cellular content of the trigger factor was reduced revealed no secretion defect for proOmpA. It has been reported that the trigger factor is also involved in GroEL-dependent protein degradation and promotes the binding of GroEL to certain protein substrates [88, 89]. The trigger factor is located close to the ribosomes and binds to the 50S subunit of the ribosome [57]. Cross-linking of the trigger factor with the nascent

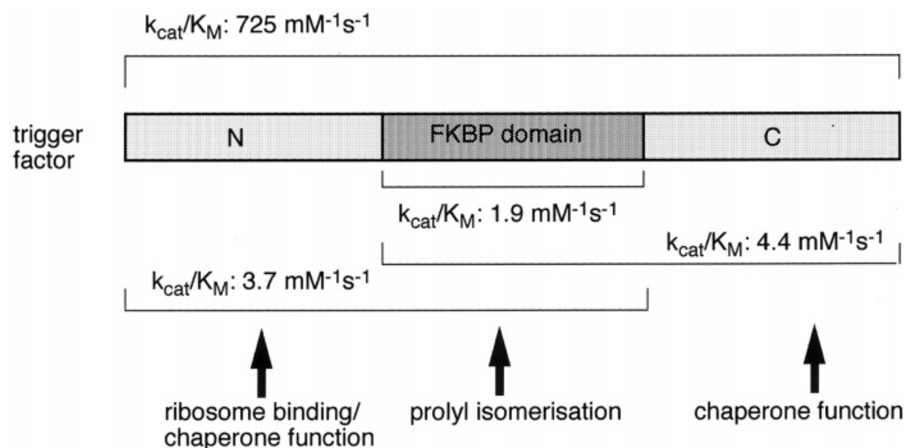

Figure 6. Schematic presentation of the trigger factor and its modular structure. The trigger factor binding to the ribosome is mediated by the N-terminal domain (N). The internal domain is the core of FKBP-type prolyl isomerase, whereas the N- and C-terminal domains are thought to bind tightly to the nascent polypeptide chain, enhancing the catalytic activity (k_{cat}/K_M) severalfold.

polypeptide chain emerging from the ribosome was shown [90].
The trigger factor has a modular structure (fig. 6) [84]. For the amino terminal, or N domain, it could be demonstrated that this domain is responsible for ribosome binding. The internal domain, or M domain, is a FKBP-type prolyl isomerase, and is the catalytic core of the trigger factor. The function of the C-terminal domain is not yet known. The high catalytic activity originates from a low K_M value, in the range of 0.5 to 1 μM, indicating that the trigger factor binds very tightly to protein substrates compared with the CyPs, FKBPs and parvulin. However, the high catalytic activity in protein folding was almost completely lost when the N-terminal and the C-terminal domains were removed from the M domain. This tight binding of protein substrates was interpreted as a chaperone-like function of the flanking domains. The high enzymatic activity in protein folding could not be restored by fusing, alternatively, the N- or the C-terminal regions to the catalytic domain (in the NM and MC constructs, respectively). The high folding activity of the intact trigger factor has been regained partially by functional complementation of the overlapping NM and MC constructs.
Together with the reduced, carboxymethylated form of the RNaseT1, the oxidized form can be used for refolding kinetics as well. If the two disulphide bonds are intact, the protein is highly stable and a largely folded intermediate is formed rapidly in refolding events. Thus the incorrect trans Tyr38-Pro39 peptidyl-prolyl bond is already partially shielded from the solvent before prolyl isomerization as the final step can take place. None of the trigger factors known so far are able to catalyze this isomerization. In contrast, small prolyl isomerases such as CyP18, FKBP12 or parvulin can recognize the largely folded intermediate and catalyze the trans to cis isomerization at Pro39 in the presence of intact disulphide bonds. However, even in the presence of prolyl isomerases (CyPs or FKBPs), the folding efficiency is poor if compared with the refolding kinetics with reduced and carboxymethylated RNaseT1.
As prolyl isomerization is often rate limiting in protein folding, and prolyl isomerases accelerate prolyl isomerization, they should be able to catalyze their own refolding in an autocatalytic manner. For a proline-limited folding reaction, autocatalysis should result in an increased folding rate with protein concentration. It has been demonstrated for the parvulins and the FKBPs that during refolding native molecules accelerate the refolding of the still unfolded molecules in an autocatalytic fashion [83].

Do prolyl isomerases act as folding catalysts in vivo?

Prolyl isomerases catalyze peptidyl-prolyl isomerization, are highly conserved from bacteria to man and are found in multiple intracellular compartments. It has been suggested therefore that they might play a critical general role in protein folding.
One well-characterized cyclophilin is the *Drosophila* NinaA protein [91]. NinaA is expressed in the eye, localized within the ER and is required for proper maturation and localization of rhodopsin. In *ninaA* mutant flies, rhodopsin accumulates in the ER, resulting in visual defects [92]. Rhodopsin and NinaA form a stable, stoichiometric complex. Flies lacking a single copy of *ninaA* exhibit defects that suggest that *ninaA* may serve as a chaperone for rhodopsin.
However, deletion of the periplasmatic cyclophilin gene from *E. coli* [93] as from *A. calcoaceticus* [94] did not show a significant phenotype in growth. Further, deletion mutants of FKBP and cyclophilin genes from many organisms did not show any significant phenotype. It was often stated that a functional overlap with unknown prolyl isomerases within one organism could suppress a significant phenotype. However, since the total genomic information is known for some organisms such as *B. subtilis* and yeast, all prolyl isomerase homologous genes of the different prolyl isomerase families are known in these organisms. Yeast contains eight cyclophilins, four FKBPs and the essential Pin1 homologue ESS1. The cyclophilin Cpr3, required for proper protein refolding after mitochondrial import [95], is necessary under elevated temperatures and for growth on lactate at 37 °C. Slower growth rates are reported for strains lacking FKBP12 and the cyclophilin40 homologue Cpr7. Surprisingly, yeast mutants lacking all 12 immunophilins were viable and the phenotype of the dodecuplet mutant is the result of simple addition of the subtle phenotypes of each individual mutation [96]. Based on these results it was concluded that the immunophilins do not play a general essential role in protein folding but rather may perform specific functions through interactions with unique sets of restricted partner proteins that remain to be identified. For the yeast FKBP12, such a partner was found recently. Aspartokinase, an enzyme catalyzing an intermediate step in threonine and methionine biosynthesis, has been identified as an in vivo binding target for FKBP12. Fpr1 mutants lacking FKBP12 are viable. They are neither threonine nor methionine auxotrophs and express wild-type levels of aspartokinase protein and activity. However, aspartokinase activity is regulated by feedback inhibition, and FKBP12 is found to be important for this feedback inhibition, possibly by catalyzing conformational changes in the asparto-

kinase in response to product binding [53]. Interestingly, for a *B. subtilis* strain lacking the trigger factor and the cyclophilin, a phenotype in growth was observed after amino acid limitation [31].

In contrast to the results mentioned above, lethal prolyl isomerase mutations have been reported in *E. coli*. A null mutation in the gene of the parvulin homologue *ppiD* leads to an overall reduction in the level and folding of outer membrane proteins in the bacterial periplasm. The combination of *ppiD* and *surA* null mutations was found to be lethal [67]. As already mentioned above, protein folding in the periplasm is thought to be different from folding in the cytosol. However, this is the first example of an essential function in general protein folding for prolyl isomerase mutations.

Conclusions

The discovery that prolyl isomerases are receptor molecules for the immunosuppressive-acting drugs CsA and FK506 led to a boom in the investigation of this class of folding catalysts. In addition to their immunosuppressive role, immunophilins act as target proteins at different cellular levels, including signal transduction, protein folding and assembly as well as cell cycle regulation. During the last few years significant progress has been made towards understanding the structure-function relationship of this ubiquitous class of proteins in vitro. However, their physiological role in vivo remains mysterious. The search for specific natural ligands of these prolyl isomerases within the cell and the construction of disruption mutations in the coding genes may provide further insights into their physiological role and their potential functions in protein folding, transport and signal transduction.

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