FEBS Letters 580 (2006) 3237–3245

Comparative analysis of enzyme activities and mRNA levels of peptidyl prolyl *cis*/*trans* isomerases in various organs of wild type and *Pin1*$^{-/-}$ mice

Jörg Fanghänel[a], Hirotada Akiyama[a,b], Chiyoko Uchida[a], Takafumi Uchida[a,b,*]

[a] *Center for Interdisciplinary Research, Graduate School of Agricultural Science, Tohoku University, 1-1 Amamiya, Tsutsumidori, Aoba, Sendai, Miyagi 981-8555, Japan*
[b] *Molecular Enzymology, Molecular Cell Science, Graduate School of Agricultural Science, Tohoku University, 1-1 Amamiya, Tsutsumidori, Aoba, Sendai, Miyagi 981-8555, Japan*

Received 6 March 2006; revised 17 March 2006; accepted 26 April 2006

Available online 6 May 2006

Edited by Judit Ovádi

Abstract **We investigated the enzyme activity of peptidyl prolyl *cis*/*trans* isomerases (PPIases) in brain, testis, lung, liver, and mouse embryonic fibroblasts (MEF) of *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ mice. The aim of this study is to determine if other PPIases can substitute for the loss of Pin1 activity in *Pin1*$^{-/-}$ mice and what influence *Pin1* depletion has on the activities of other PPIases members. The results show that high PPIase activities of Pin1 are found in organs that have the tendency to develop *Pin1* knockout phenotypes and, therefore, provide for the first time an enzymological basis for these observations. Furthermore we determined the specific activity (k_{cat}/K_M) of endogenous Pin1 and found that it is strongly reduced as compared with the recombinant protein in all investigated organs. These results suggest that posttranslational modifications may influence the PPIase activity in vivo. The activities originating from cyclophilin and FKBP are not influenced by the Pin1 knockout, but a basal enzymatic activity towards phosphorylated substrates could be found in *Pin1*$^{-/-}$ lysates. Real time PCR experiments of all PPIases in different mouse organs and MEF of *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ mice support the finding and reveal the specific expression profiles of PPIases in mice.**

Keywords: PPIase; Pin1; Real time PCR; Fkbp6; Fkbp7

1. Introduction

Peptidyl prolyl *cis*/*trans* isomerases (PPIases) accelerate the *cis*/*trans* isomerization of peptidyl prolyl bonds in peptides and proteins both in vivo and in vitro [1–3]. According to their sequence homologies and sensitivities to specific inhibitors, PPIases can be divided into three families, cyclophilin, FKBP, and parvulins. Pin1 belongs to the parvulin family. It is the only PPIase known to have a high specificity to substrates with phosphorylated serine and threonine side chains preceeding proline [4,5]. Pin1 consists of a PPIase and a WW domain which interact with phosphorylated (pS)P- and phosphothreonine (pT)P-motifs in peptides and proteins [5,6].

Pin1 controls the functions of phosphorylated proteins, such as cell cycle regulatory molecules: CDC25C [7], Wee1 [8], Myt1 [9], CK2 [10], Plk1 [11], tis21 [12], cyclin D1 [13], Raf-1 [14]; transcription factors: NFAT [15], c-Jun [16], c-Fos [17], p54nrb [18], the hSpt5 subunit of the DRB sensitivity-inducing factor [19], C-terminal domain of RNA polymerase II [20]; tumor suppressor proteins: p53 [17], p73 [21]; steroid receptors: SRC-3 [22]; mouse embryonic midline and left/right axial development: Sil [23]; and chromatin condensation: topoisomerase IIα [10].

Although the depletion of Pin1 in *Saccharomyces cervices* and *Candida albicans* caused cell death [24,25], mice, [26] *Drosophila melanogaster* [26,27] and a strain of *S. cervices C110* [28] lacking Pin1 were viable. The phenotypes in *Pin1*$^{-/-}$mice include a higher risk of developing Alzheimer's disease [29], testicular and retinal atrophies, and that the breast epithelial compartment failed to undergo the normal changes associated with pregnancy [13,27]. In other organs such as lung and liver, no pathological changes have been observed so far. It has been proposed that *Pin1*$^{-/-}$ mice are viable because other PPIases might act for Pin1 and thereby help the organisms to develop. Cyclophilin A and parvulin 14, the second parvulin-type of PPIases, are slightly overexpressed in Pin1-depletion yeast and mouse embryonic fibroblasts (MEF), respectively [28,30]. In the human genome, a *Pin1* homologue has been identified which is about 90% identical to *Pin1* [31]. The corresponding mouse protein with the same high level of identity has not been investigated, but no cross reactions of polyclonal Pin1 antibodies could be observed in *Pin1*$^{-/-}$ mouse tissues [27], questioning the expression of this gene in mice.

This article describes the analysis of all cyclophilin- and FKBP-type PPIases known in mice, and compares the mRNA levels and activities of PPIases in organs that show a strong phenotype, such brain and testis, with organs having no pronounced phenotypes, such as lung and liver.

2. Materials and methods

Substrates were obtained from Bachem (Weil am Rhein, Germany). Cyclosporine A was purchased from Wako Pure Chemicals, Ltd (Japan). FK506 was a gift from Astellas Pharma Inc. (Tokyo, Japan). Chymotrypsin and trypsin were purchased from Sigma–Aldrich (Tokyo, Japan).

*Corresponding author. Fax: +81 22 717 8778.
E-mail address: uchidat@biochem.tohoku.ac.jp (T. Uchida).

Abbreviations: pS, phosphoserine; pT, phosphothreonine; PPIase, peptidyl prolyl *cis*/*trans* isomerase

0014-5793/$32.00
doi:10.1016/j.febslet.2006.04.087

2.1. *Pin1*$^{-/-}$ mice and MEF

Pin1-KO mice were created from the 129SV ES cells and then the Pin1 gene deletion was transferred into an isogenic C57BL/6J background. The wild type mice were obtained from the littermates. MEF were produced and cultured as previously described [27,30].

2.2. RNA preparation, cDNA synthesis, and real time PCR analysis

Total RNA from 100 to 200 mg of tissue was prepared by using Sepazol RNA extraction solution (Nakalai Tesque Inc., Kyoto, Japan) following the protocol provided by the manufacturer. To increase RNA purity, the extraction protocol was repeated. The quality of the total RNA was checked by denaturing agarose gel electrophoresis containing 1.5% formaldehyde. Total RNA concentration and purity was determined by UV–Vis spectroscopy with the Bio-Rad SmartSpec 3000 system (California, USA). cDNA synthesis was performed by using TaqMan reverse transcription reagents (Applied Biosystems, New Jersey, USA) as instructed by the manufacturer. All sequences of the primer pairs used for real time PCR were obtained from the PrimerBank [32]. Real Time PCR was performed with the 7300 Real Time PCR System (Applied Biosystems, Warrington, UK). Total sample volume was 25 μl. The real time PCR protocol consisted of two initial steps (50 °C for 2 min, followed by 94 °C for 5 min), 40 cycles of DNA amplification (94 °C for 1 min, 57 °C for 45 s, 72 °C for 45 s) and a PCR-product dissociation stage for quality control. The quality of the PCR products was further checked by 2.5% agarose gel electrophoresis. In all cases, single bands of the expected size were observed. Relative transcript quantities were calculated as ΔCT values, as recommended by the manufacturer with GAPDH as the endogenous reference amplified from the samples.

2.3. Preparation of protein samples for Western blot and PPIase assay

Freshly prepared mouse organs were cut into small pieces (approx. 50–100 mg) and frozen in liquid nitrogen. Frozen tissues were then thawed in 500 μl ice cold NP40 buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% (v/v) NP40, 5 mM EDTA, 1 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4) supplemented with protease inhibitor cocktail (Nakalai Tesque, Inc. Kyoto, Japan). The material was homogenized by ultra-turrax T8 (IKA, Staufen, Germany) for 15 s at the highest speed setting. The lysate was diluted with NP40 buffer to a total volume of 2.5 ml. Immediately after dilution, the lysate was divided into aliquots of 50 μl, subsequently frozen in liquid nitrogen, and stored until used at −80 °C. Insoluble parts in the lysates were removed before use by centrifugation of the thawed sample for 20 s at 21 000 × *g*. The protein concentration of the samples was determined by using the BCA Protein Assay Kit (Pierce, Illinois, USA) according to the manufacturer's protocol. The total protein concentrations for all samples were between 2 and 7 mg/ml.

2.4. PPIase assay

The PPIase assay was performed by the protease-coupled assay described earlier [1,33]. Total sample volume was 1500 μl, sample buffer was 35 mM HEPES, pH 7.5. Stock solutions of all substrates (30 mg/ml) were prepared in 0.47 M LiCl/TFE (anhydrous). Stock solutions of proteases (100 mg/ml for chymotrypsin and 50 mg/ml for trypsin) were in 35 mM HEPES, pH 7.8. Insoluble materials from all stock solutions were removed by centrifugation before use.

To measure the total cyclophilin-related PPIase activity, the substrate Suc-AAPF-pNA was used. In addition to the substrate (0.03 mg/ml) and protease (chymotrypsin, 0.2 mg/ml), a final concentration of 33 μM FK506 was present during the measurement to inhibit FKBP-related activities. The FKBP activity was measured with the substrate Suc-ALPF-pNA (0.03 mg/ml) in the presence of chymotrypsin (0.2 mg/ml) and 33 μM cyclosporine A. To determine the PPIase activity originating from Pin1, the phosphorylated substrate Ac-AA(pS)PR-pNA (0.02 mg/ml) in combination with trypsin (0.1 mg/ml), 33 μM cyclosporine A and 33 μM FK506 was employed. For a typical measurement, the sample buffer was incubated in a temperature-controlled cuvette holder at 10 °C for 4 min. After the inhibitors and substrate were mixed, the reaction was started by the injection of the protease. After the initial burst phase where the all-*trans* peptides were cleaved, the protein sample was added to the reaction. The reaction was recorded with an Agilent 8453 UV–Vis spectrophotometer (California, USA), and the signal difference between 390 and 510 nm was used to calculate the first-order rate constants.

3. Results

3.1. PPIase activity against phosphorylated substrates

Knockout of *Pin1* in mice results in organ-specific phenotypes. Physiological and histological cellular changes are observed in the brain, testis, and ovary; whereas, other organs do not show noticeable effects [13,27,34]. To investigate whether these different effects of Pin1 are due to a specific backup by other PPIases, the PPIase activities in brain, testis, lung, and liver and an embryonic cell line were compared in WT and *Pin1*$^{-/-}$ mice.

The observed enzymatic Pin1 activities varied strongly within the investigated organs (Fig. 2). The PPIase activity was normalized to the total protein concentration of each respective lysate. The activity is shown in arbitrary units, calculated by using the observed first-order rate constant of the enzymatically catalyzed reaction (k_{obs}) subtracting the rate constant of the uncatalyzed reaction (k_{uncat}) and dividing that value by the total protein concentration of the lysate in the sample. Almost no Pin1 activity was found in liver and lung; whereas, the PPIase activity of Pin1 in brain and testis was substantial. To prove that the observed activity is exclusively produced by Pin1, we analyzed cell lysates obtained from *Pin1*$^{-/-}$ mouse tissues. No PPIase activity was found when the phosphorylated substrate was used with FK506 and cyclosporine A in the reaction mixture. From this result, the question arises as to how the enzymatic activity of Pin1 is regulated. The same lysates as prepared for the activity assays were used to determine the amount of Pin1 by Western blot (Fig. 2A). The absolute amount of endogenous Pin1 in the lysate was determined by comparing the intensity of the Pin1 band with the bands from known amounts of recombinant Pin1 on the same Western blot gel. This enabled us to estimate the specific activity of Pin1 in the different tissues. As shown in Fig. 2B, the k_{cat}/K_M values of Pin1 were very similar in all tissues. We also checked the PPIase activity against the phosphorylated substrate without the addition of CsA or FK506. The lack of FK506 did not change the activity levels in the WT and *Pin1*$^{-/-}$ mouse lysates, although the activity was increased when CsA was not added into the mixture. These results were observed to the same extent in the WT and *Pin1*$^{-/-}$ lysates.

Interestingly, the specific activity of Pin1 in the lysates was about 4–9 times lower than that found for the recombinant protein (Fig. 3). The WT mouse lysates included the natural ligands of Pin1, which could act as a competitive inhibitor to the in vitro substrate used for the activity assays. We therefore checked whether incubation of the recombinant GST-Pin1 with *Pin1*$^{-/-}$ MEF cell lysates reduced the specific activity. The observed 50% reduction of the specific activity (Fig. 3) can be explained by the high ionic content of the lysis buffer used.

3.2. PPIase activities of cyclophilins and FKBPs

The cyclophilin-related PPIase activity in the investigated tissues is quite equally distributed (Fig. 4). The use of the Suc-AAPF-pNA substrate in combination with a high concentration of FK506 ensured that no activity originating from FKBP or Pin1 inferred with the measurement. The highest activity was found in MEF and Brain tissue. The activity profile of FKBP was similar to that of the cyclophilin-related activity (Fig. 5), with the difference that the highest activity was found in testis lysates. The small differences found in the activity levels between WT and *Pin1*$^{-/-}$ are within the range of error, which is

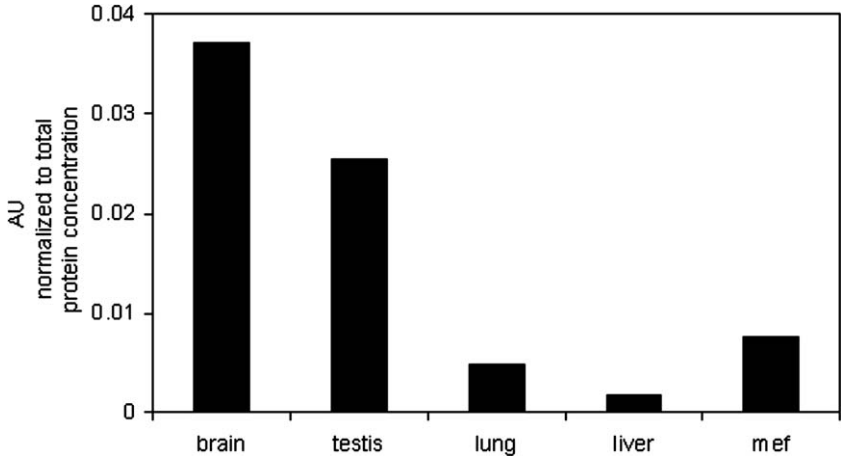

Fig. 1. PPIase activity of Pin1 against Ac-AA(pS)PR-pNA substrate measured in tissue lysates of brain, testis, lung, liver, and MEF. The measured activity was normalized to the total protein concentration of the lysate ($n = 4$). To avoid any influence from cyclophilins and FKBPs present in the lysate, 33 μM cyclosporine A and 33 μM FK506 were added to all reactions.

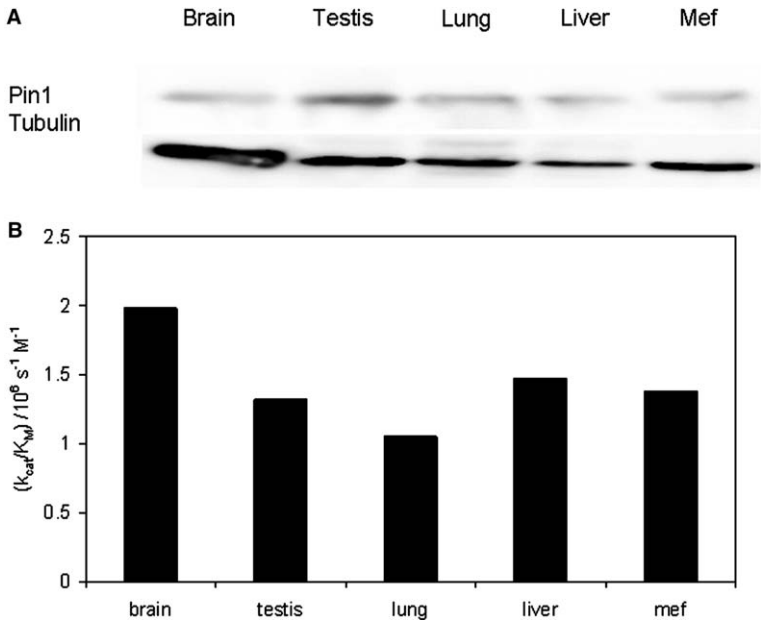

Fig. 2. Pin1 activity in tissue lysates of brain, testis, lung, liver, and MEF normalized to the total amount of endogenous Pin1 in the lysates. The expression of Pin1 in these tissues was shown by immunoblot (A). The second-order rate constants were calculated by dividing the first-order rate constant k_{enz} by the Pin1 concentration present in the assay (B). The error of the values is the combination of the error made in estimating the absolute Pin1 protein amount by Western blot (S.E.M.; ±30%, $n = 4$) and the error range of the PPIase assay (S.E.M.; ±10%, $n = 4$).

a combination of the error made by estimating the total protein concentration (S.E.M. = ±20%) and the error made in determining the PPIase activity (S.E.M. = ±10%). The major difference between the cyclophilin and FKBP activity levels in the different tissues is the approximately 20- to 30-fold higher cyclophilin-related activity.

3.3. mRNA level of PPIases in mouse organs

We determined the mRNA levels of all individual FKBPs and cyclophilins in Table 1 to ensure that the similar observed PPIase activities in *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ lysates are not a direct consequence of a simultaneous upregulation of one set of PPIases compensated for by a downregulation of another set of PPIases.

In Figs. 7 and 8, the overall expressions of cyclophilins and FKBPs are shown for four different WT organs (brain, testis, lung, and liver) and an MEF cell line normalized to the expression of the endogenous control GAPDH. The expression levels of PPIases Nos. 16, 30, and 31 were below the detection limit of the method for all organs and were therefore excluded. No PPIases were exclusively expressed highly in lung and liver as could be expected for a PPIase that specifically substitutes Pin1, and thereby represses the Pin1-specific phenotype in these organs. We also checked the mRNA level of a Pin1L a gene with very high homology to Pin1, which was suspected to be responsible for the lack of phenotype in *Pin1*$^{-/-}$ *D. melanogaster* and mice [31], but no expression in any organ could be found. It shows that this

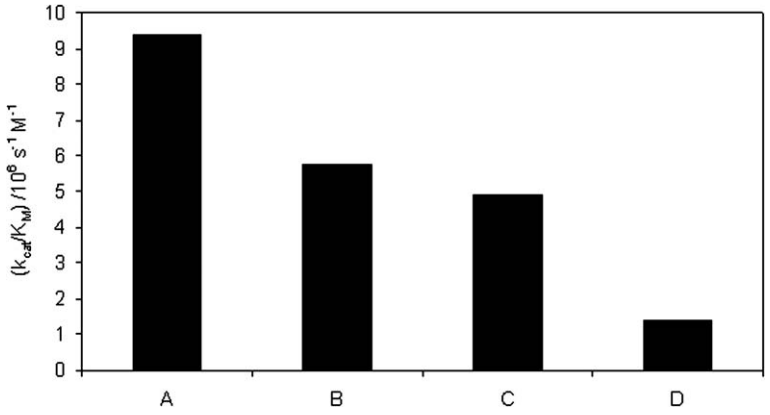

Fig. 3. Pin1 activity of GST-Pin1 (A) compared with the activity of GST-Pin1 preincubated in *Pin1*$^{-/-}$ MEF lysates (B) or in lysis buffer plus 0.1 mg BSA (C). For easier comparison, the activity of endogenous Pin1 in MEF is also included (D). The error of the values (A–C) is the combination of the error of estimating the absolute Pin1 protein by UV–Vis spectroscopy (S.E.M.; ±10%, $n = 4$) and the error range of the PPIase assay (S.E.M.; ±10%, $n = 4$).

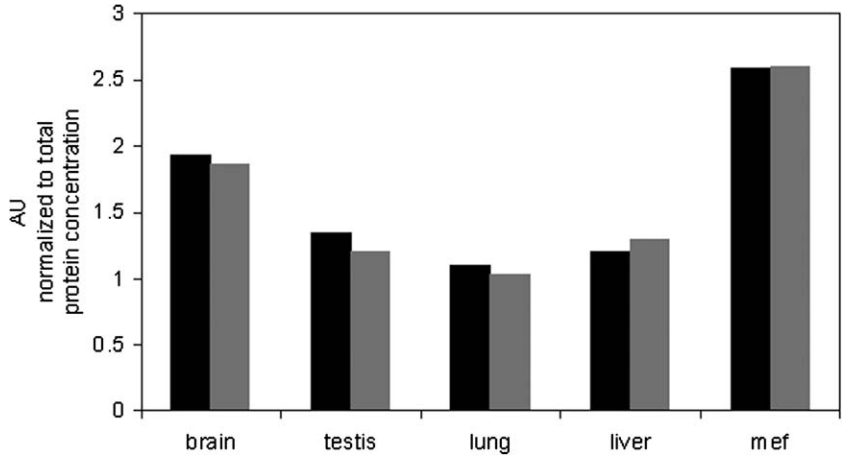

Fig. 4. Comparison of the PPIase activity of cyclophilins against Suc-AAPF-pNA substrate measured in tissue lysates of brain, testis, lung, liver, and MEF of Pin1 WT and *Pin1*$^{-/-}$. The measured activity was normalized to the total protein concentration of the lysate ($n = 4$). To avoid any influence from FKBPs present in the lysate, 33 μM FK506 was added to all reactions.

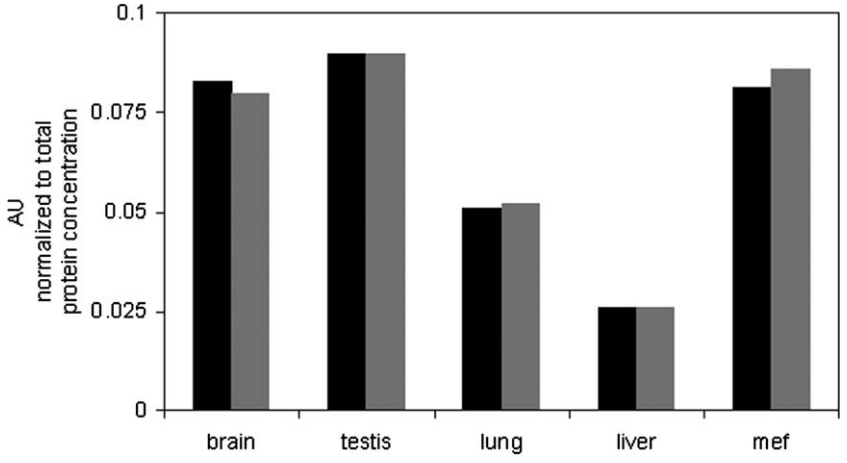

Fig. 5. Comparison of the PPIase activity of FKBPs against Suc-ALPF-pNA substrate measured in tissue lysates of brain, testis, lung, liver, and MEF of Pin1 WT and *Pin1*$^{-/-}$. The measured activity was normalized to the total protein concentration of the lysate ($n = 4$). To avoid any influence from cyclophilins present in the lysate, 33 μM cyclosporine A was added to all reactions.

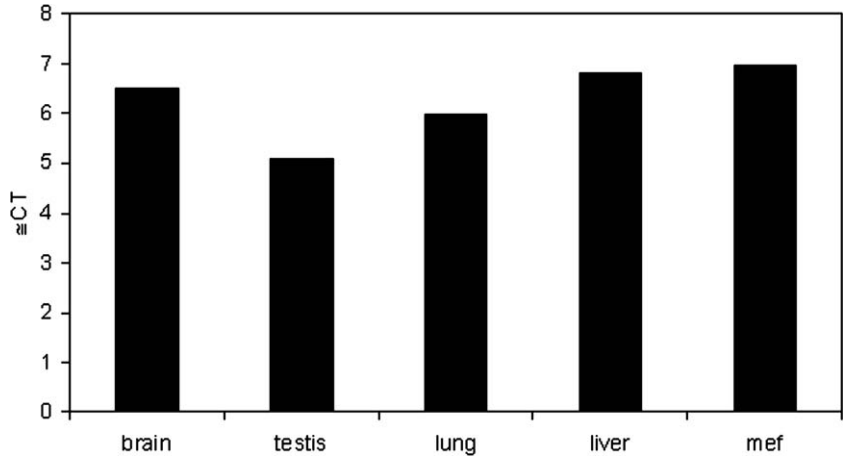

Fig. 6. Real time PCR analysis of Pin1 in brain, testis, lung, liver, and MEF. CT-values were normalized to the expression of the endogenous GAPDH control ($n = 3$).

Table 1
Swissprot entries of PPIases in mouse

Cyclophilin		FKBP	
No.	Swissprot/Gen name	No.	Swissprot/Gen name
1	P17742/*Ppia*	17	P26883/*Fkbp1a*
2	Q99KR7/*Ppif*	18	Q9Z2I2/*Fkbp1b*
3	Q9QZH3/*Ppie*	19	P30416/*Fkbp4*
4	Q9ERU9/*Ranbp2*	20	Q64378/*Fkbp5*
5	P24369/*Ppib*	21	P45878/*Fkbp2*
6	Q9CR16/*Ppid*	22	Q62446/*Fkbp3*
7	P30412/*Ppic*	23	P59024/*Fkbp14*
8	Q9D868/*Ppih*	24	Q61576/*Fkbp10*
9	Q13427/*Ppig*	25	Q9Z247/*Fkbp9*
10	P30415/*Nktr*	26	Q9D1M7/*Fkbp11*
11	Q9D0W5/*Ppil1*	27	Q91XW8/*Fkbp6*
12	Q812D2/*Ppil3*	28	O54998/*Fkbp7*
13	Q9D787/*Ppil2*	29	O35465/*Fkbp8*
14	Q9D3J1/*Ppil4*	30	O08915/*Aip*
15	Q9D6D8/*Ppil6*	31	Q924K1/*Aipl*
16	AK014025[a]		

[a]GenBank Accession Number.

gene is not expressed in mice and can, therefore, be classified as a pseudogene.

Despite the differences in other activities, the mRNA level of *Pin1* is quite similar in all investigated organs (Fig. 6). The maximal difference of $\Delta\Delta CT = 1.9$ can be found between testis and MEF.

In Fig. 9, the average $\Delta\Delta CT$ values for FKBP in WT brain compared to *Pin1*$^{-/-}$ brain are exemplarily shown. Three different mice were used to prepare mRNA from whole brain tissue. The CT values are normalized to the endogenous GAPDH level. The only FKBP with a $|\Delta\Delta CT|$ larger than 2 is *Fkbp6* ($\Delta\Delta CT = 2.2$). A $\Delta\Delta CT$ value of 2 corresponds to a 4-fold higher level of expression in the *Pin1*$^{-/-}$ brain. The total amount of *Fkbp6* mRNA in the brain (Fig. 8) is very low. Accordingly, the amount of Fkbp6 protein in the brain is below the detection level of the available antibody (data not shown) [35]. In addition to *Fkbp6* expression in the brain, *Fkbp7* in primary MEF cells shows a higher expression level in the knockout cells, with a $\Delta\Delta CT$ value of 2.8. This difference translates to an approximately 7-fold increase in mRNA in

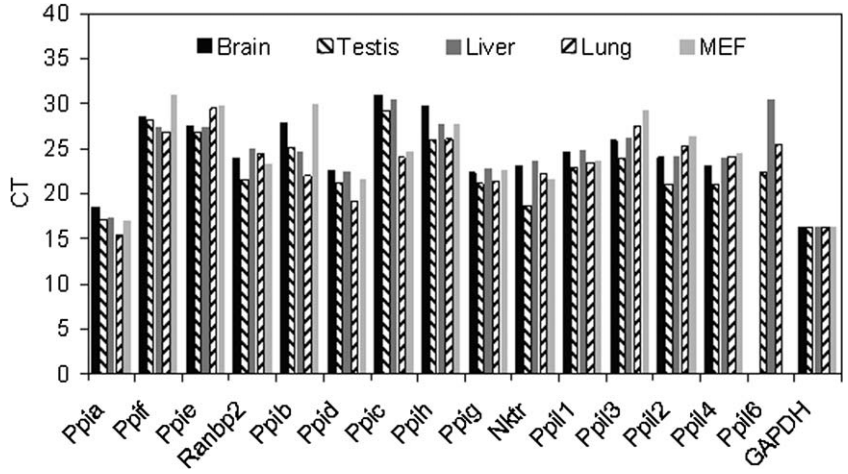

Fig. 7. Real time PCR analysis of cyclophilins in brain, testis, lung, liver, and MEF. CT-values were normalized to the expression of the level of the endogenous GAPDH control in MEF ($n = 3$).

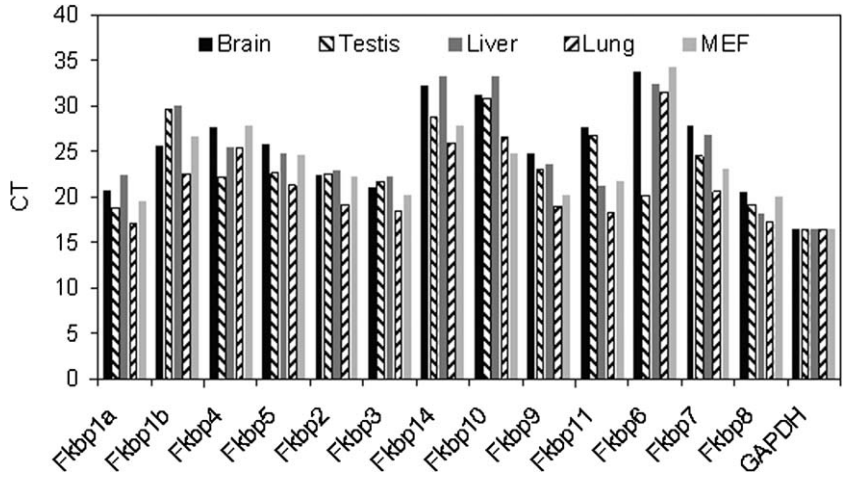

Fig. 8. Real time PCR analysis of FKBPs in brain, testis, lung, liver, and MEF. CT-values were normalized to the expression of the level of the endogenous GAPDH control in MEF ($n = 3$).

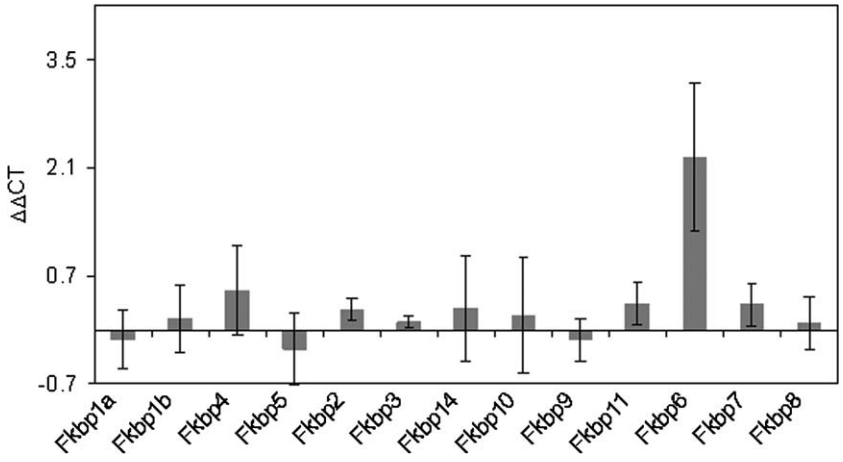

Fig. 9. ΔΔCT analysis of the mRNA level of FKBPs in brain normalized to the expression of the endogenous GAPDH control ($n = 3$).

Pin1$^{-/-}$ MEF. All other PPIases in all other tissues did not show a change in the $|\Delta\Delta CT|$ values larger than 2.

Not all Pin1-related physiological effects are exclusively mediated through its PPIase domain [10,15,36]. We therefore checked the mRNA levels of proteins possessing a WW domain with comparable substrate specificity to Pin1. According to the consensus sequence postulated [37–39], five proteins in mice were found to have such a (pT/pS)P-specific WW domain (Table 2). Real time PCR experiments showed, however, that none of these proteins have a difference in $|\Delta\Delta CT|$ values between WT and *Pin1*$^{-/-}$ tissues larger than 2.

Table 2
Swissprot entries of proteins containing a WW domain with a similar substrate specificity as the Pin1-WW domain

No.	Swissprot/Gen name
1	Q91wl8/Wwox
2	Q8C7V0/1300018I05Rik
3	Q9JHC1/Fnbp4
4	P46935/Nedd4
5	P59114/Pcif1

3.4. Structural comparison

The finding that two FKBPs are more highly expressed in *Pin1*$^{-/-}$ tissues as compared with their WT counterparts led us to investigate the structural similarities of Pin1 and FKBP in more detail. We used the available mammalian FKBP structures found in the RCSB-databank.

By superimposing the central β2 and β6 β-sheets in the FKBP domains with the two central β-sheets of the Pin1 (β1 and β4) PPIase domain and the small α-helix positioned over the active sites (α1 and α4 for FKBP and Pin1, respectively) we could check whether a cluster of positively charged side chains can be found in the respective FKBP in a position close to the β1/α1 phosphate/sulfate binding loop of Pin1. We found a high similarity between the phosphate specificity determining loop in Pin1 and the loop connecting the β5 and β6 sheets of the second FKBP domain of Fkbp5 (FKBP51) and Fkbp4 (FKBP52) (Fig. 10). The finding that this loop in the named FKBP is capable of binding negatively charged ligands is underlined by the fact that in the crystal structure of FKBP51, a sulfate ion is bound at this position (PDB code: 1KTO) [40]. Homology searches revealed that this positive charged patch is also present in FKBP14.

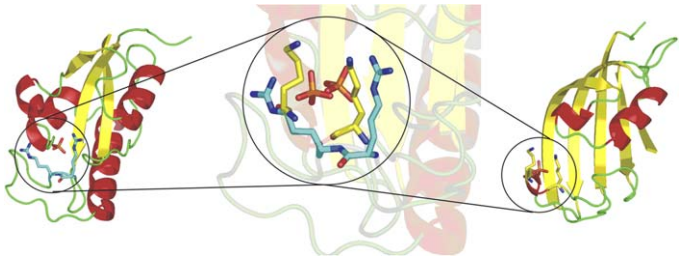

Fig. 10. Structural comparison of Pin1 (left, pdb code: 1Pin) and the second PPIase domain of FKBP51 (right, pdb code: 1Kto). The Structures were superimposed by aligning the central β2, β6 β-sheets in the second FKBP domain of Fkbp6 with the two central β-sheets of the Pin1 (β1 and β4) PPIase domain; additionally, the small α-helix positioned over the active sites (α1 and α4 for Fkbp6 and Pin1, respectively) of both enzymes were also included in the alignment. The superimposition (middle) shows the two arginine residues of Pin1 (R68 and R69) and the two lysine residues of Fkbp6 (K230 and K232), in complex with their respective sulfate ions present in the crystal structure.

3.5. Discussion

An earlier work showed that in primary MEF a second member of the parvulin type family (parvulin 14) had slightly higher mRNA and protein levels in *Pin1*$^{-/-}$ MEF cells [30]. In contrast to the brain and testis tissues of *Pin1*$^{-/-}$ mice, primary MEF cells show only a moderate phenotype [27], and, therefore, might not be suitable for investigating the question whether other PPIases act in the place of Pin1 in *Pin1*$^{-/-}$ mice. We have therefore compared the PPIase activities between WT and *Pin1*$^{-/-}$ mice, in those organs that develop *Pin1*$^{-/-}$ related phenotypes and those without phenotypes.

The most striking observation was that even though the mRNA level of Pin1 is similar in the organs that show phenotypes in the *Pin1*$^{-/-}$ mice (e.g., brain and testis, and to a lesser extent MEF) and the organs without phenotypes (lung and liver) the Pin1 activity was very different (Fig. 1). High Pin1 activity levels were only found in those organs that show significant phenotypes in *Pin1*$^{-/-}$ mice. Almost no Pin1 PPIase activity was observed in organs such as the liver and lung where no *Pin1*$^{-/-}$phenotypes have been reported. Even though the total activity is much higher in brain, testis, and to some extent in MEF, the specific activity is very similar in all organs. The results show that the observed differences are a direct consequence of the higher proportion of Pin1 compared with the total protein (Fig. 2). These results suggest that Pin1 activity is mainly controlled by its expression level but not by the modification. This finding provides the first enzymologically based explanation for the organ specificity of the Pin1 phenotypes.

Until now, the means whereby the Pin1 function is regulated has not been well known. It has been shown, however, that the WW domain can be phosphorylated at a serine in position 16, and as a consequence the domain is unable to bind to its native substrates [36]. The phosphorylation at serine 65 in the PPIases domain, on the other hand, does not impair the PPIase function [11]. Our results clearly show that the PPIase activity of endogenous Pin1 is lower than that of recombinant Pin1 (Fig. 3). This effect was observed to the same extent in all tissues. The reduced activity is not caused by natural Pin1 ligands, which could compete with the in vitro substrate, since the activity of recombinant Pin1 incubated with *Pin1*$^{-/-}$ lysates does not produce such results. Amazingly, even though the Pin1-specific activity (k_{cat}/K_M) is reduced in the organs, the total activity is only between 3- to 10-fold lower than the combined activity of the FKBPs. In contrast, cyclophilin-related activity is about 30-fold higher than the FKBP activity. This difference can be explained by two facts: first, the specific activities of most cyclophilin-type PPIases are at least 10-fold higher than those for FKBPs (for overview see [41]); and, second, some FKBPs' PPIase activities are highly regulated and might, therefore, exist in their inactive state in the lysate [42]. In general it was observed that neither the PPIase activity originated by FKBP nor by cyclophilins was changed by the *Pin1* knockout (Figs. 4, 5).

The broader substrate specificity of the cyclophilins allows the catalysis of phosphorylated substrates [4]. We could observe that cyclosporine A inhibits all PPIase activity measured with the phosphorylated substrate in *Pin1*$^{-/-}$ lysates. The same increase in activity was measured with this substrate in WT and *Pin1*$^{-/-}$ lysates, if no cyclosporin A was added to the reaction mixture. It can be concluded that the total activity of cyclophilins is unchanged in the *Pin1*$^{-/-}$ lysates when they are measured with the phosphorylated substrate. This is in agreement with the observation that the total cyclophilin activities as well as the mRNA levels of these enzymes are not changed as a result of the *Pin1* knockout (Figs. 4, 7). The total cyclophilin activities of the different tissues do not show a strong variance; nevertheless, the phenotypes are present in some but not all tissues. This fact argues against the possibility that cyclophilins specifically substitute for Pin1 activity. But, the basal activity towards phosphorylated substrates could be responsible for the general viability of the different cell-types. It has been already reported that extremely small amounts of ESS1 are sufficient to overcome the lethal phenotype of the ESS1 knockout in yeast [43]. Such a small amount of Pin1 activity could similarly be mimiced by cyclophilins.

Since, in the presence of cyclosporine A, no activity could be found with the phosphorylated substrate in *Pin1*$^{-/-}$ lysates, we could conclude that under the conditions used here, FKBPs are not able to catalyze the *cis* to *trans* isomerization of this substrate. Yet, we can not entirely exclude the idea that the FKBP activity is only observable with the native in vivo substrates or that the activity in general might be too small to be detected by our methods while being high enough to substitute for Pin1.

Only the total activity of FKBPs and Cyclophilins can be tested with this assay. We found that the mRNA levels of most PPIases are unchanged for both *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ animals, excluding the possibility that the lack of activity differences between WT and knockout mice is accidentally caused by a simultaneous up and down regulation of different PPIases. The real time PCR profiles show that no PPIases are exclusively more highly expressed in lung, liver, and MEF, as one

would expect, if PPIases specifically prevented the development of Pin1 knockout phenotypes. The higher expression levels of Fkbp6 found in the brain of *Pin1*$^{-/-}$ and Fkbp7 in the MEF of *Pin1*$^{-/-}$ animals have no obvious effects on the PPIase activity against in vitro substrates. In the case of Fkbp6, this could be explained by the lower expression level of this protein in the brain (Fig. 8). Interestingly, despite the lack of any sequence homology, a striking resemblance between the tertiary protein structures of the FKBP and the parvulin family has previously been observed [5,44]. Furthermore, it was observed that many essential active side residues are conserved between parvulins and FKBP [41,45]. Here we could find that the loop between the β5 and β6 sheets of the second FKBP domains of Fkbp5 and Fkbp4 (FKBP51/52) and FKBP14 shows a high similarity to the phosphate specificity determining loop of Pin1. These findings open a new way to view FKBPs and their possible function in catalyzing the peptidyl prolyl *cis*/*trans* isomerization in phosphorylated substrates; these enzymes might, in contrast to cyclophilins, be highly specific for such substrates. The FKBP4, FKBP5 and FKBP14 have a sequence that could be the equivalent of the phosphate binding loop of PIN1. That should be proven with recombinant enzyme and phosphorylated substrates.Further investigations will clarify our hypothesis.

To summarize our results and earlier observations, it seems that the lethal phenotype related to the control of the cell cycle of Pin1 observed in single cell organisms can be overcome by a basal level of PPIase activity of cyclophilins or by a specific increase in the expression of certain FKBPs (such as Fkbp6 and Fkbp7) in higher organisms. The structural similarities of FKBP51/52 and FKBP also open the possibility that these PPIases could catalyze phosphorylated substrates. To carry out such functions, very low amounts of Pin1 and, therefore, very little PPIase activity towards ligands with (pS/pT)-P motifs have been shown to be sufficient. In organs, such as the brain and testis, where higher levels of Pin1 activity can be found, this basal activity is not sufficient to substitute for the missing Pin1 activity resulting in the development of Pin1-specific phenotypes.

Acknowledgements: We acknowledge Makoto Suzuki, Hiroyuki Mizukami, Shinsaku Ootaki and Katsuyoshi Kamiie for technical help. We thank Junko Noguchi for providing the antibody against Fkbp6. This work was supported in part by a Grant of Specially Promoted Research of Center for Interdisciplinary Research, Tohoku University, a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Grant-in-Aid for Scientific Research on Priority Areas.

References

[1] Fischer, G., Bang, H. and Mech, C. (1984) Determination of enzymatic catalysis for the *cis–trans*-isomerization of peptide binding in proline-containing peptides. Biochim. Biophys. Acta 43, 1101–1111.
[2] Lang, K., Schmid, F.X. and Fischer, G. (1987) Catalysis of protein folding by prolyl isomerase. Nature 329, 268–270.
[3] Bosco, D.A. and Kern, D. (2004) Catalysis and binding of cyclophilin A with different HIV-1 capsid constructs. Biochemistry 43, 6110–6119.
[4] Yaffe, M.B., Schutkowski, M., Shen, M., Zhou, X.Z., Stukenberg, P.T., Rahfeld, J.U., Xu, J., Kuang, J., Kirschner, M.W., Fischer, G., Cantley, L.C. and Lu, K.P. (1997) Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. Science 278, 1957–1960.
[5] Ranganathan, R., Lu, K.P., Hunter, T. and Noel, J.P. (1997) Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent. Cell 89, 875–886.
[6] Verdecia, M.A., Bowman, M.E., Lu, K.P., Hunter, T. and Noel, J.P. (2000) Structural basis for phosphoserine-proline recognition by group IV WW domains. Nat. Struct. Biol. 7, 639–643.
[7] Crenshaw, D.G., Yang, J., Means, A.R. and Kornbluth, S. (1998) The mitotic peptidyl-prolyl isomerase, Pin1, interacts with Cdc25 and Plx1. EMBO J. 17, 1315–1327.
[8] Shen, M., Stukenberg, P.T., Kirschner, M.W. and Lu, K.P. (1998) The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins. Genes Dev. 12, 706–720.
[9] Wells, N.J., Watanabe, N., Tokusumi, T., Jiang, W., Verdecia, M.A. and Hunter, T. (1999) The C-terminal domain of the Cdc2 inhibitory kinase Myt1 interacts with Cdc2 complexes and is required for inhibition of G(2)/M progression. J. Cell Sci. 112 (Pt19), 3361–3371.
[10] Messenger, M.M., Saulnier, R.B., Gilchrist, A.D., Diamond, P., Gorbsky, G.J. and Litchfield, D.W. (2002) Interactions between protein kinase CK2 and Pin1. Evidence for phosphorylation-dependent interactions. J. Biol Chem. 277, 23054–23064.
[11] Eckerdt, F., Yuan, J., Saxena, K., Martin, B., Kappel, S., Lindenau, C., Kramer, A., Naumann, S., Daum, S., Fischer, G., Dikic, D., Kaufmann, M. and Strebhardt, K. (2005) Polo-like kinase 1-mediated phosphorylation stabilizes Pin1 by inhibiting its ubiquitination in human cells. J. Biol. Chem. 280, 36575–36583.
[12] Hong, J.W., Ryu, M.S. and Lim, I.K. (2005) Phosphorylation of serine 147 of tis21/BTG2/pc3 by p-Erk1/2 induces Pin-1 binding in cytoplasm and cell death. J. Biol. Chem. 280, 21256–21263.
[13] Liou, Y.C., Ryo, A., Huang, H.K., Lu, P.J., Bronson, R., Fujimori, F., Uchida, T., Hunter, T. and Lu, K.P. (2002) Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. Proc. Natl. Acad. Sci. USA 99, 1335–1340.
[14] Dougherty, M.K., Muller, J., Ritt, D.A., Zhou, M., Zhou, X.Z., Copeland, T.D., Conrads, T.P., Veenstra, T.D., Lu, K.P. and Morrison, D.K. (2005) Regulation of Raf-1 by direct feedback phosphorylation. Mol. Cell. 17, 215–224.
[15] Liu, W., Youn, H.D., Zhou, X.Z., Lu, K.P. and Liu, J.O. (2001) Binding and regulation of the transcription factor NFAT by the peptidyl prolyl *cis–trans* isomerase Pin1. FEBS Lett. 496, 105–108.
[16] Wulf, G.M., Ryo, A., Wulf, G.G., Lee, S.W., Niu, T., Petkova, V. and Lu, K.P. (2001) Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing the transcriptional activity of c-Jun towards cyclin D1. EMBO J. 20, 3459–3472.
[17] Monje, P., Hernandez-Losa, J., Lyons, R.J., Castellone, M.D. and Gutkind, J.S. (2005) Regulation of the transcriptional activity of c-Fos by ERK. A novel role for the prolyl isomerase PIN1. J. Biol. Chem. 280, 35081–35084.
[18] Proteau, A., Blier, S., Albert, A.L., Lavoie, S.B., Traish, A.M. and Vincent, M. (2005) The multifunctional nuclear protein p54nrb is multiphosphorylated in mitosis and interacts with the mitotic regulator Pin1. J. Mol. Biol. 346, 1163–1172.
[19] Lavoie, S.B., Albert, A.L., Handa, H., Vincent, M. and Bensaude, O. (2001) The peptidyl-prolyl isomerase Pin1 interacts with hSpt5 phosphorylated by Cdk9. J. Mol. Biol. 312, 675–685.
[20] Albert, A., Lavoie, S. and Vincent, M. (1999) A hyperphosphorylated form of RNA polymerase II is the major interphase antigen of the phosphoprotein antibody MPM-2 and interacts with the peptidyl-prolyl isomerase Pin1. J. Cell Sci. 112 (Pt 15), 2493–2500.
[21] Mantovani, F., Piazza, S., Gostissa, M., Strano, S., Zacchi, P., Mantovani, R., Blandino, G. and Del Sal, G. (2004) Pin1 links the activities of c-Abl and p300 in regulating p73 function. Mol. Cell. 14, 366–625.
[22] Yi, P., Wu, R.C., Sandquist, J., Wong, J., Tsai, S.Y., Tsai, M.J., Means, A.R. and O'Malley, B.W. (2005) Peptidyl-prolyl isomerase 1 (Pin1) serves as a coactivator of steroid receptor by regulating the activity of phosphorylated steroid receptor coactivator 3 (SRC-3/AIB1). Mol. Cell. Biol. 25, 9687–9699.
[23] Campaner, S., Kaldis, P., Izraeli, S. and Kirsch, I.R. (2005) Sil phosphorylation in a Pin1 binding domain affects the duration of the spindle checkpoint. Mol. Cell. Biol. 25, 6660–6672.

[24] Hanes, S.D., Shank, P.R. and Bostian, K.A. (1989) Sequence and mutational analysis of ESS1, a gene essential for growth in *Saccharomyces cerevisiae*. Yeast 5, 55–72.
[25] Devasahayam, G., Chaturvedi, V. and Hanes, S.D. (2002) The Ess1 prolyl isomerase is required for growth and morphogenetic switching in *Candida albicans*. Genetics 160, 37–48.
[26] Maleszka, R., Hanes, S.D., Hackett, R.L., de Couet, H.G. and Miklos, G.L. (1996) The *Drosophila melanogaster* dodo (dod) gene, conserved in humans, is functionally interchangeable with the ESS1 cell division gene of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 93, 447–451.
[27] Fujimori, F., Takahashi, K., Uchida, C. and Uchida, T. (1999) Mice lacking Pin1 develop normally, but are defective in entering cell cycle from G(0) arrest. Biochem. Biophys. Res. Commun. 265, 658–663.
[28] Fujimori, F., Kikuchi, J., Gunji, W., Mogi, T., Hatta, Y., Makino, T., Okuhara, K., Uchida, T. and Murakami, Y. (2001) Crosstalk of prolyl isomerases, Pin1/Ess1, and cyclophilin A. Biochem. Biophys. Res. Commun. 289, 181–190.
[29] Liou, Y.C., Sun, A., Ryo, A., Zhou, X.Z., Yu, Z.X., Huang, H.K., Uchida, T., Bronson, R., Bing, G., Li, X., Hunter, T. and Lu, KP. (2003) Role of the prolyl isomerase Pin1 in protecting against age-dependent neurodegeneration. Nature 424, 556–561.
[30] Uchida, T., Takamiya, M., Takahashi, M., Miyashita, H., Ikeda, H., Terada, T., Matsuo, Y., Shirouzu, M., Yokoyama, S., Fujimori, F. and Hunter, T. (2003) Pin1 and Par14 peptidyl prolyl isomerase inhibitors block cell proliferation. Chem. Biol. 10, 15–24.
[31] Campbell, H.D., Webb, G.C., Fountain, S. and Young, I.G. (1997) The human PIN1 peptidyl-prolyl *cis*/*trans* isomerase gene maps to human chromosome 19p13 and the closely related PIN1L gene to 1p31. Genomics 44, 157–162.
[32] Wang, X. and Seed, B. (2003) A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res. 31, e154.
[33] Kofron, J.L., Kuzmic, P., Kishore, V., Colon-Bonilla, E. and Rich, D.H. (1991) Determination of kinetic constants for peptidyl prolyl *cis–trans* isomerases by an improved spectrophotometric assay. Biochemistry 30, 6127–6134.
[34] Lu, P.J., Wulf, G., Zhou, X.Z., Davies, P. and Lu, K.P. (1999) The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein. Nature 399, 784–788.
[35] Crackower, M.A. et al. (2003) Essential role of Fkbp6 in male fertility and homologous chromosome pairing in meiosis. Science 300, 1291–1295.
[36] Lu, P.J., Zhou, X.Z., Liou, Y.C., Noel, J.P. and Lu, K.P. (2002) Critical role of WW domain phosphorylation in regulating phosphoserine binding activity and Pin1 function. J. Biol. Chem. 277, 2381–2384.
[37] Otte, L., Wiedemann, U., Schlegel, B., Pires, J.R., Beyermann, M., Schmieder, P., Krause, G., Volkmer-Engert, R., Schneider-Mergener, J. and Oschkinat, H. (2003) WW domain sequence activity relationships identified using ligand recognition propensities of 42 WW domains. Protein Sci. 12, 491–500.
[38] Schleinkofer, K., Wiedemann, U., Otte, L., Wang, T., Krause, G., Oschkinat, H. and Wade, R.C. (2004) Comparative structural and energetic analysis of WW domain–peptide interactions. J. Mol. Biol. 344, 865–881.
[39] Kato, Y., Ito, M., Kawai, K., Nagata, K. and Tanokura, M. (2002) Determinants of ligand specificity in groups I and IV WW domains as studied by surface plasmon resonance and model building. J. Biol. Chem. 277, 10173–10177.
[40] Sinars, C.R., Cheung-Flynn, J., Rimerman, R.A., Scammell, J.G., Smith, D.F. and Clardy, J. (2003) Structure of the large FK506-binding protein FKBP51, an Hsp90-binding protein and a component of steroid receptor complexes. Proc. Natl. Acad. Sci. USA 100, 868–873.
[41] Fanghanel, J. and Fischer, G. (2004) Insights into the catalytic mechanism of peptidyl prolyl *cis*/*trans* isomerases. Front. Biosci. 9, 3453–3478.
[42] Edlich, F., Weiwad, M., Erdmann, F., Fanghanel, J., Jarczowski, F., Rahfeld, J.U. and Fischer, G. (2005) Bcl-2 regulator FKBP38 is activated by Ca^{2+}/calmodulin. EMBO J. 24, 2688–2699.
[43] Gemmill, T.R., Wu, X. and Hanes, S.D. (2005) Vanishingly low levels of Ess1 prolyl-isomerase activity are sufficient for growth in *Saccharomyces cerevisiae*. J. Biol. Chem. 280, 15510–15517.
[44] Sekerina, E., Rahfeld, J.U., Muller, J., Fanghanel, J., Rascher, C., Fischer, G. and Bayer, P. (2000) NMR solution structure of hPar14 reveals similarity to the peptidyl prolyl *cis*/*trans* isomerase domain of the mitotic regulator hPin1 but indicates a different functionality of the protein. J. Mol. Biol. 301, 1003–10017.
[45] Jacobs, D.M., Saxena, K., Vogtherr, M., Bernado, P., Pons, M. and Fiebig, K.M. (2003) Peptide binding induces large scale changes in inter-domain mobility in human Pin1. J. Biol. Chem. 278, 26174–26182.