FKBP36 Forms Complexes with Clathrin and Hsp72 in Spermatocytes[†]

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ABSTRACT: The testes-specific peptidyl-prolyl *cis*/*trans* isomerase FKBP36 plays a crucial role in male meiosis. Here we show that the catalytic domain of FKBP36 binds to clathrin heavy chain (CHC) of clathrin. Despite wild-type FKBP36 not displaying PPIase activity, the introduction of the R81L substitution resulted in catalysis of prolyl isomerization, which is comparable to the regulated activity of FKBP38. Furthermore, the TPR domain of FKBP36 interacts with Hsp72. In fact, FKBP36 preferentially binds to Hsp72 among the members of the Hsp70 family and is thus the first TPR-containing protein which discriminates between Hsp70 proteins. The clathrin−FKBP36−Hsp72 complexes resulting from both identified interactions are bound to the matrices of clathrin-coated vesicles in spermatocytes, which indicates a possible role of FKBP36 and Hsp72 in the disassembly of clathrin coats.

FKBP36 belongs to the peptidyl-prolyl *cis*/*trans* isomerase (PPIase,[1] EC 5.2.1.8) family of FK506-binding proteins (FKBPs). The FKBP6 gene, which encodes FKBP36, was shown to be involved in Williams-Beuren syndrome (WBS) (*1*). FKBP6 belongs to a cluster of genes on chromosome 7, which is completely or in part deleted in WBS patients. The dominant autosomal disorder WBS is characterized by supravalvular aortic stenosis, multiple peripheral pulmonary arterial stenoses, elfin face, mental and statural deficiency, characteristic dental malformation, and infantile hypercalcemia (*2*). The functional role of FKBP36 was studied previously by generating a FKBP36-deficient mouse (*3*). The absence of FKBP36 in rodents resulted in aspermic males and the absence of normal pachytene spermatocytes. In contrast, female FKBP6 −/− mice are fertile and perfectly normal. Crackower et al. identified FKBP36 as a component of the synaptonemal complex, where this FKBP localizes to meiotic chromosome cores and regions of homologous chromosome synapsis.

FKBP36 features two domains: (i) an N-terminal PPIase domain of the FKBP type and (ii) a C-terminal protein interaction domain containing three tetratrico peptide repeat (TPR) motifs. FKBPs usually catalyze the isomerization of peptide bonds preceding the amino acid proline in polypeptides (*4*). Moreover, these enzymes are characterized by their ability to bind tightly to the immunosuppressive drugs FK506 and rapamycin. The complexes of FKBPs and both low-molecular weight inhibitors are known to be involved in immunosuppression (*5*).

Several human FKBPs feature TPR motifs, which are degenerative sequences of 34 amino acids mediating interprotein interactions (*6*). Members of the FKBP family bind with their TPR domains to the C-terminal domains of heat shock proteins, such as Hsp90 (*7*). These interactions are involved in multiprotein complex formation. Complexes between dimers of Hsp90 and either FKBP51 or FKBP52 have been reported to interact with client molecules, e.g., steroid hormone receptors, to modify the activity, stability, or subcellular localization of the client proteins (*7*). Within these complexes, the FKBPs interact with both the receptor and Hsp90 (*8*).

Here we show that the PPIase domain of FKBP36 interacts with clathrin, in a manner independent of the putative active site of FKBP36. Additionally, a second interaction occurs between the FKBP36 TPR domain and the C-terminal domain of Hsp72 that leads to the formation of clathrin−FKBP36−Hsp72 complexes. These complexes are present at the matrix of clathrin-coated vesicles of rat testes cells.

EXPERIMENTAL PROCEDURES

Sources of Enzymes. Human FKBP36, human Hsp72 C10, human Hsp71 C10, and human Hsc70 C10 were employed; human Hsp70L C10 and human Hsp90 C90 were recombinantly expressed by using a pET28a vector in *Escherichia coli* Rosetta cells. GST fusion FKBP36 variants were expressed using a pGEX4T1 vector in *E. coli* Rosetta cells. Rat and bovine testis CHC was purified from clathrin-coated vesicles. Chymotrypsin was purchased from Sigma. The FKBP36 antibody is a polyclonal rabbit antibody against purified FKBP36. In addition, we used polyclonal rabbit anti-actin (Sigma), monoclonal mouse anti-$(His)_5$ (Qiagen), monoclonal mouse anti-GST (Sigma), monoclonal rat anti-Hsp90 (StressGene), polyclonal rabbit anti-Hsp72 (StressGene), polyclonal rabbit anti-GAPDH (Sigma), and polyclonal rabbit anti-H3 (Cell Signaling Technology, Danvers,

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[1] Abbreviations: FKBP, FK506-binding protein; PPIase, peptidyl-prolyl *cis*/*trans* isomerase; CHC, clathrin heavy chain; Hsp, heat shock protein; WBS, Williams-Beuren syndrome; TPR, tetratrico peptide repeat; CsA, cyclosporine A.

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MA) antibodies. The PPIase substrates (Suc-Ala-Xaa-Pro-Phe-pNA) were purchased from Bachem (Heidelberg, Germany). FK506 was purchased from Calbiochem (La Jolla, CA).

PPIase Activity Assay. Measurements were performed in 35 mM Hepes buffer (pH 7.8) at 10 °C in the protease-coupled assay (*24*). Peptide substrates of the type succinyl-Ala-Xaa-Pro-Phe-4-nitroanilide, where Xaa represents a variable amino acid position N-terminal of the amino acid proline, were dissolved in dimethyl sulfoxide. A Hewlett-Packard 8452 diode array UV−vis spectrophotometer was used for monitoring the time course of the reaction. The obtained first-order kinetics can be described by the rate equation $v = k_{obs}[cis]$; $k_{obs} = k_0 + k_{enz}$ ([*cis*] is the time-dependent concentration of the *cis* conformer, k_0 is the uncatalyzed, and k_{enz} is the rate of the PPIase-catalyzed *cis*−*trans* interconversion of the substrate). The specificity constant k_{cat}/K_M was obtained from the equation $k_{cat}/K_M = (k_{obs} - k_0)/[PPIase]$, assuming that the entire amount of the enzyme represents catalytically active molecules. Inhibition constants for the PPIase activity of FKBP36 and FKBP36 R81L by FK506 were determined by a competition assay using recombinant FKBP12 and the succinyl-AFPF-4-nitroanilide substrate (*11*).

Fluorescence Spectroscopy. Fluorescence spectra were recorded with a Perkin-Elmer FluoroMax2 fluorescence spectrometer, using a 1 cm × 1 cm cuvette, an excitation wavelength of 280 nm, and excitation and emission slit widths of 5 and 3 nm, respectively. Protein samples were applied in 10 mM Tris buffer (pH 7.5).

Protein Interaction Assays. For the GSH-Sepharose binding assay, 30 μL of GSH-Sepharose was pre-equilibrated in 25 mM Tris-HCl buffer (pH 7.5) (150 mM NaCl and 1 mM DTT) and saturated with GST or GST fusion proteins. Then the beads were washed twice with buffer B [25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM DTT] and subsequently incubated for 2 h with 100 μL of rat testes cytoplasm. In some experiments, 50 μM Hsp90 C90 or 50 μM Hsp72 C10 was added. Beads were centrifuged, washed three times, and mixed with an equal volume of Laemmli sample buffer. Samples were then subjected to 10% SDS−PAGE and analyzed by Western blotting.

Preparation of Subcellular Fractions. Subcellular fractions were prepared from either fresh or frozen rat testis. Rat testis were comminuted in 10 mM Hepes-NaOH (pH 7.2), 0.15 M NaCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM DTT, 1 μg/mL pepstatin A, 1 μg/mL leupeptin, and 0.2 mM PMSF, followed by homogenization in a potter homogenizer. The crude extract was centrifuged for 10 min at 1000*g*. The pellet was used for the preparation of nuclei (*25*). The supernatant was centrifuged again for 15 min at 15000*g* and for 45 min at 100000*g* to prepare cytosol and membrane fractions. Membrane fractions were either extracted with 0.5 M Tris (pH 7.5) to produce clathrin-coated vesicle components or with 1% Triton. The membrane fraction was used for preparation of clathrin-coated vesicles (*26*).

Sucrose Gradient Centrifugation. For sedimentation analysis, clathrin-coated vesicles were subjected to a 0 to 60% sucrose gradient centrifugation at 38000 rpm for 1 h at 4 °C in an SW41 Ti rotor (Beckman).

Dissociation of Clathrin-Coated Vesicles and Preparation of CHC. For dissociation of coat proteins, crude clathrin-coated vesicles were resuspended in 0.5 M Tris-HCl (pH 7.4). After incubation at room temperature for 30 min, these mixtures were centrifuged at 100000*g* for 1 h. Samples of the supernatant and pellet were collected for protein analysis. For the preparation of purified CHC, the supernatant was subjected to gel filtration in 0.5 M Tris-HCl (pH 7.4) (Pharmacia S200 16/60 column). CHC-containing fractions were pooled and centrifuged at 100000*g* for 1 h, and clathrin trimers were concentrated by addition of ammonium sulfate to 50% saturation.

RESULTS

PPIase Activity of FKBP36. The multidomain FKBP36 was considered as FKBP on the basis of the similarity of its protein sequence to the sequences of several members of this PPIase family (*1*). The N-terminal FKBP36 domain, comprising Gly^{37}−Leu^{143}, features 35−45% identical residues in the corresponding positions of the PPIase domains of other human FKBPs, including FKBP12 and FKBP52. FKBP36 has a domain composition similar to those of FKBP37.7, FKBP44, and FKBP38, the sequence of which is 45% identical or similar with that of FKBP36.

Fourteen FKBP12 residues are involved in the formation of a hydrophobic pocket, which comprises the active site and mediates FK506 binding with high affinity (*9, 10*). The PPIase domain of FKBP36 features eight conserved and two similar residues in the corresponding positions. Thus, the FKBP36 activity was tested in standard PPIase assays. However, neither the full-length protein nor the isolated PPIase domain of FKBP36 possessed catalytic activity in the presence of different peptide substrates (data not shown). In addition, the capability of FKBP36 to bind to FK506 was analyzed using a FKBP competition assay (*11*), but in analogy to the PPIase measurements, no FK506 binding of FKBP36 was observed.

Further analysis of the FKBP36 residues corresponding to the active site residues of FKBP12 revealed a significant difference between both proteins. Arg^{81} of FKBP36 corresponds to Phe^{46} of FKBP12 and probably protrudes with a positive charge in the largely hydrophobic cavity. This difference in wild-type (WT) FKBP36 might interfere with the binding of peptide substrates. Hence, we introduced the R81F substitution into FKBP36 to investigate the influence of the arginine side chain on FKBP36 activity, but in contrast to WT FKBP36, the expression of this variant in *E. coli* resulted in an insoluble protein. However, the FKBP36 R81L variant carrying a hydrophobic leucine, as present in the corresponding position of FKBP25, was expressed in the soluble fraction of *E. coli* and had a content of secondary structure elements and stability similar to those of the WT protein.

Interestingly, the FKBP36 R81L variant exhibited catalytic activity in the PPIase assay, which revealed a k_{cat}/K_M value of $(5.9 \pm 0.2) \times 10^3\ M^{-1}\ s^{-1}$ for succinyl-Ala-Phe-Pro-Phe-*p*-nitroanilide substrate. This catalytic efficiency is comparable to the activity of the CaM-Ca^{2+}-activated FKBP38 and significantly lower than those of constitutively active FKBPs (*11, 12*). Interestingly, FKBP36 R81L activity was not inhibited by either the FKBP inhibitors FK506 and rapamycin or the cyclophilin inhibitor CsA (data not shown). Measurements using different tetrapeptide substrates showed

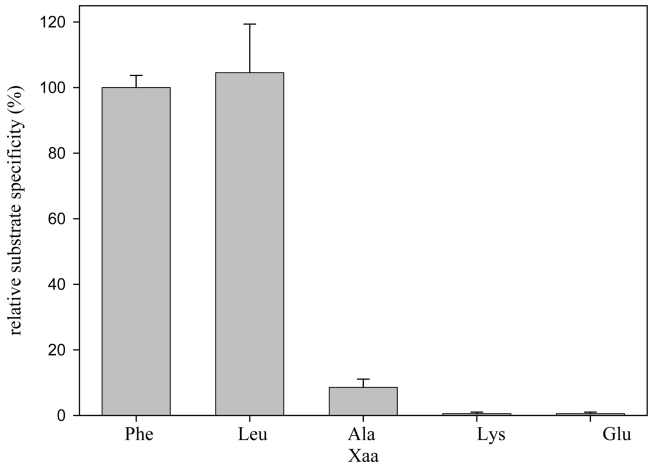

FIGURE 1: Substrate specificity of FKBP36 R81L. The ratios of the specificity constants k_{cat}/K_m for peptide substrates of the type Suc-Ala-Xaa-Pro-Phe-4-nitroanilide are shown. PPIase activity against the Suc-Ala-Phe-Pro-Phe-4-nitroanilide substrate was set to 100%.

that the FKBP36 R81L variant has a unique substrate specificity, which is, however, similar to other FKBP substrate specificities (Figure 1). The FKBP36 R81L variant preferred the bulky hydrophobic residues leucine and phenylalanine and did not accept substrates with charged amino acid side chains, like glutamate and lysine, in the position N-terminal to proline. This substrate specificity excludes the possibility that the detected enzymatic activity is caused by an *E. coli* PPIase, which theoretically could be present as an impurity with the recombinant protein.

These results demonstrate that the lack of catalytic activity of the FKBP36WT variant is based on the interference of the Arg^{81} side chain with the binding of peptide substrates. Arg^{81} is conserved in vertebrate orthologs of FKBP36, but not found in the corresponding positions of other human FKBPs. Thus, a conserved participation of the FKBP36 active site in a signal transduction pathway and specific interaction partners of the catalytic domain of FKBP36 can be inferred. Furthermore, FK506 binding to FKBP36 is not prevented by Arg^{81}, because the FKBP36 R81L variant is also not targeted by the low-molecular weight inhibitor, which adds to the unique features of the FKBP36 active site.

The PPIase Domain of FKBP36 Interacts with CHC of Clathrin. To identify novel interaction partners of FKBP36, a glutathione *S*-transferase fusion protein was coupled to a GSH affinity matrix and applied to rat testes lysate. The experiments showed interactions between FKBP36 and a protein, which was identified by MALDI-TOF analysis as CHC. Thus, GST-bound FKBP36 was incubated with purified CHC to test the presence of direct interactions between both proteins. Figure 2A shows that CHC bound to the GST−FKBP36 fusion protein, whereas no interactions with only GST were observed.

To further probe the interaction between clathrin and different FKBP36 variants, we determined whether clathrin interacts with the PPIase domain of FKBP36. In fact, CHC bound to GST-fused full-length FKBP36 and the $FKBP36^{1-142}$ variant, whereas no binding to the GST−$FKBP36^{145-327}$ protein was observed (Figure 2B). Moreover, the $FKBP36^{37-327}$ variant lacking the N-terminal extension bound to CHC in a manner comparable to that of the wild-type protein. Thus, CHC binds to the PPIase domain of FKBP36, and the C-terminal TPR domain of FKBP36 is not involved in clathrin binding.

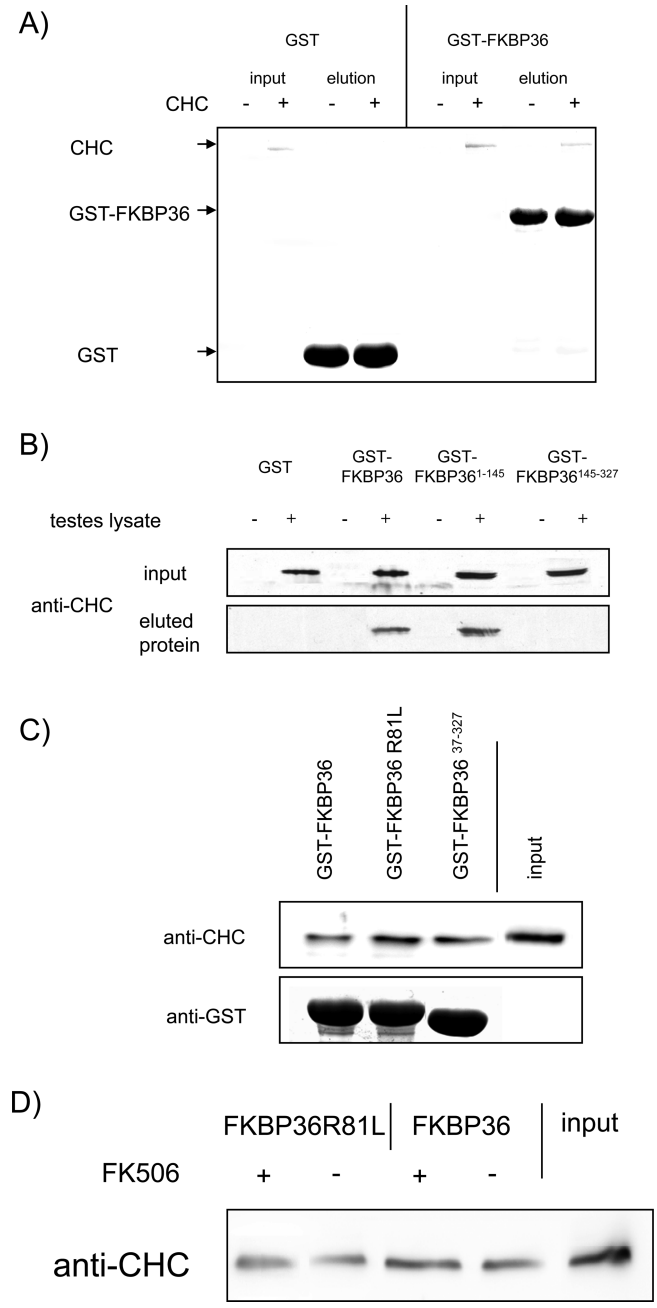

FIGURE 2: FKBP36 interacts directly with CHC. (A) Coomassie-stained SDS−PAGE (10%) of samples of an incubation experiment using purified clathrin trimer from rat testis and the GST−FKBP36 protein- or GST-preloaded GSH-Sepharose. Samples without CHC served as the control. (B) GSH-Sepharose preloaded with either GST−FKBP36 variants or only GST was incubated with rat testes lysate and subsequently analyzed by SDS−PAGE and Western blotting using anti-CHC. (C) Rat testes lysate was incubated with GSH-Sepharose preloaded with different GST−FKBP36 variants and analyzed by SDS−PAGE and Western blotting using anti-CHC and anti-GST antibodies. (D) GST−FKBP36 variants were loaded on GSH-Sepharose and incubated with rat testes lysate either in the presence or in the absence of FK506 and subsequently analyzed by SDS−PAGE and Western blotting using anti-CHC antibodies.

Consequently, the influence of the clathrin triskelion on the activity of WT FKBP36 and FKBP36 R81L was assayed, but the measured prolyl isomerization in the presence of

either WT FKBP36 or FKBP36 R81L was not influenced by clathrin trimers (data not shown). However, clathrin showed interactions with the FKBP36 R81L variant similar to those of the wild-type protein (Figure 2C). Furthermore, we tested the influence of FK506 on the interaction between clathrin and FKBP36 (Figure 2D). The interaction between both proteins was not influenced by FK506, which is in agreement with the findings that (i) FK506 does not inhibit FKBP36 and (ii) the clathrin triskelion has no influence on FKBP36 activity. These results demonstrate that clathrin binds to the PPIase domain of FKBP36, but no interactions between the active site of FKBP36 and CHC are involved.

FKBP36 Is a Component of Clathrin-Coated Vesicles. To analyze cellular interactions between FKBP36 and clathrin, a subcellular fractionation of rat testes cells was performed. FKBP36 was found to localize in the nucleus and cytoplasm, as previously reported (*3*). Figure 3A shows that in addition a significant fraction of endogenous FKBP36 was extracted from membranes with 0.5 M Tris, which is typical for components of clathrin-coated vesicles.

In a further attempt to analyze whether the interaction partners are found colocalized in the cell, the subcellular compartments were further fractionated by differential centrifugation. Figure 3B shows a similar pattern for clathrin and FKBP36, showing that both interaction partners are similarly distributed in the compartments of rat testes cells. In addition, a significant population of Hsp72 was detected to colocalize with FKBP36 and CHC in the fraction of clathrin-coated vesicles. Moreover, purified vesicles from rat testes were subjected to a sucrose gradient centrifugation to analyze the association of FKBP36 with the clathrin coat. After fractionation, the distribution of different proteins was subjected to Western blot analysis. CHC was identified in fractions 6−27 with a peak in fraction 15, and β-adaptins were found in fractions 9−27 (Figure 3C). A similar distribution in the sucrose gradient was observed for the endogenous FKBP36. Moreover, copurified FKBP36 is found in the supernatant after disassembly of the matrix of clathrin-coated vesicles (Figure 3D). The FKBP36 pattern is, thus, similar to the results found for clathrin.

Collectively, these findings show that FKBP36 binds to matrix proteins of clathrin-coated vesicles. In pull-down experiments in which purified clathrin-coated vesicles are loaded onto a GST−FKBP36 affinity matrix, in addition to CHC, β-adaptins and Hsp72 were identified binders of the FKBP36 matrix (Figure 3E). Analogous experiments with dissociated clathrin-coated vesicles resulted in binding of clathrin trimers and Hsp72 to the FKBP36 affinity matrix, whereas β-adaptins did not bind (Figure 3E). Hence, FKBP36 interacts directly with clathrin and Hsp72 in the matrix of clathrin-coated vesicles. The interaction among FKBP36, clathrin, and Hsp72 is independent of the assembly of the clathrin triskelion to clathrin-coated vesicles.

FKBP36 Discriminates between Different Hsp70 Proteins. Since FKBP36 interacts with clathrin and Hsp72, we analyzed whether CHC and Hsp72 compete for the same binding site in FKBP36. To this end, we applied Hsp72 to different FKBP36 variants with GST fusion proteins loaded on GSH-Sepharose. Hsp72 bound specifically to affinity matrices loaded with WT FKBP36 and the FKBP36$^{145-327}$ variant that comprises the C-terminal TPR domain of FKBP36 (Figure 4A).

TPR domains are known to mediate the interaction with the C-terminal domains of either Hsp70 proteins or Hsp90. Thus, the purified C-terminal Hsp72 domain was used in a competition experiment with endogenous proteins interacting with a FKBP36 affinity matrix (Figure 4B). Indeed, the purified C-terminal Hsp72 domain competed with endogenous Hsp72, demonstrating an interaction between FKBP36 and the C-terminal domain of Hsp72. Moreover, the C-terminal Hsp72 domain competed also with Hsp90 for FKBP36 binding, which clearly shows that both heat shock proteins bind to FKBP36 in a similar manner. These observations were confirmed by a competition of the C-terminal Hsp90 domain with both Hsp72 and Hsp90 from rat testes lysate. Hence, FKBP36 forms complexes with clathrin and either Hsp72 or Hsp90.

Hsp72 is not the only member of the Hsp70 family that is expressed in testes cells (*13*). Hence, we performed a pull-down experiment using purified C-terminal domains of Hsp90, Hsp72, Hsp70c, Hsp71, and Hsp70L to investigate the possibility of selective interactions between FKBP36 and the different members of the Hsp70 family. Figure 5A shows that the C-terminal domains of Hsp90 and Hsp72 bind to the FKBP36 affinity matrix as in previous experiments. The C-terminal domains of other Hsp70 family members, however, did not bind to FKBP36 in significant amounts. Thus, FKBP36 discriminates between the Hsp70 proteins and binds preferentially to Hsp72. These results were also confirmed by fluorescence measurements (Figure 5B). Only the presence of the C-terminal Hsp72 domain changed the protein fluorescence of FKBP36 significantly.

DISCUSSION

The testis-specific FKBP36 is essential in male fertility and has apparently several interaction partners, which indicate different physiological functions of this multidomain PPIase. Our results demonstrate that these interactions are mediated, in part, by the catalytic domain, even though the putative active site is probably not involved and WT FKBP36 lacks PPIase activity against standard tetrapeptide substrates.

In contrast, the FKBP36 R81L variant exhibited catalytic activity and unique substrate specificity. Thus, the catalytic domain of FKBP36 is, in principle, able to catalyze the *cis*−*trans* isomerization of prolyl bonds in polypeptide chains, but the presence of the Arg^{81} side chain in the putative active side interferes with the PPIase activity of FKBP36. Consequently, FKBP36 either has substrates that differ greatly from the peptides that were used or requires prior activation by an unknown mechanism.

In the absence of a physiological substrate peptide, one could only speculate that FKBP36 substrates may not even involve proline residues, if FKBP36 is constitutively active. Peptide bond isomerase activity against substrates without proline residues has been described previously as a feature of secondary amide bond isomerases (*14*). However, FKBP36 could also be regulated by an unknown mechanism and thus be involved in a specific signal transduction pathway. FKBP38, which has the sequence most similar to that of FKBP36 among the human FKBPs, is inactive in its free form like FKBP36, but FKBP38 becomes activated upon binding to CaM-Ca^{2+} and then participates in neuronal apoptosis control by inhibition of Bcl-2 (*11*). Interestingly,

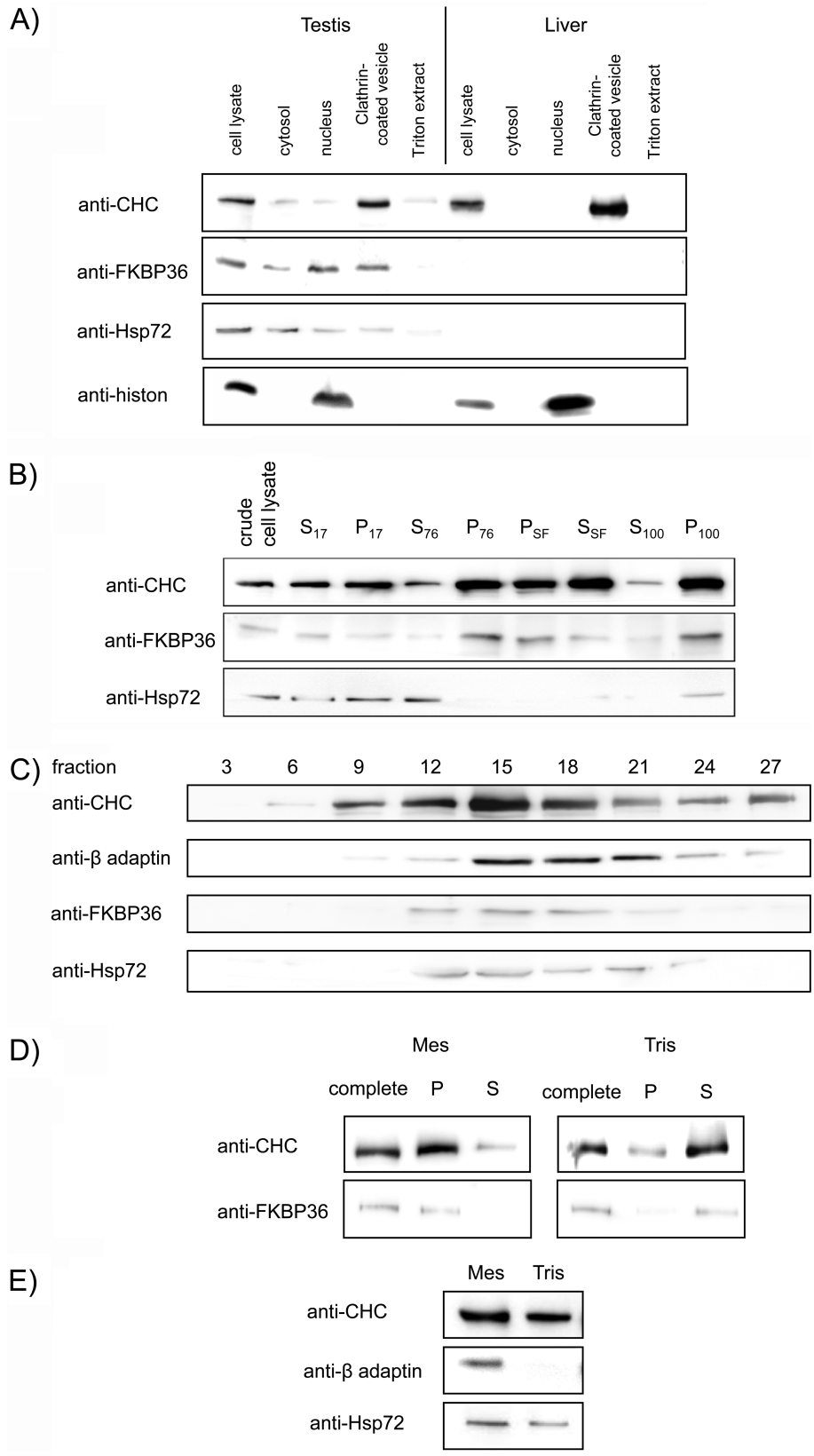

FIGURE 3: FKBP36 is a component of clathrin-coated vesicles. (A) Western blot analysis of a subcellular fractionation of either rat testis cells or rat liver using anti-CHC, anti-FKBP36, anti-GAPDH, and anti-histone antibodies. (B) Western blot analysis of a clathrin-coated vesicle preparation from rat testis lysate by differential centrifugation. The supernatant (S) and pellet (P) of sedimentation steps at 17000*g*, 76000*g*, and 100000*g* and accordingly a sucrose−ficoll gradient (SF) were analyzed using anti-CHC, anti-Hsp72, and anti-FKBP36 antibodies. (C) Fractions of a sucrose gradient centrifugation (from 0 to 60% sucrose) of purified clathrin-coated vesicles were analyzed by Western blotting using anti-β-adaptin, anti-CHC, and anti-FKBP36 antibodies. (D) Sedimentation analysis of FKBP36 and CHC from either assembled or dissociated clathrin-coated vesicles. The supernatant (S) and pellet (P) from an ultracentrifugation were analyzed by Western blotting using anti-CHC and anti-FKBP36 antibodies. (E) GST−FKBP36 protein-loaded GSH-Sepharose was incubated with either assembled (Mes) or dissociated (Tris) clathrin-coated vesicles. The presence of CHC, β-adaptin, and Hsp72 in the eluate was analyzed by Western blotting.

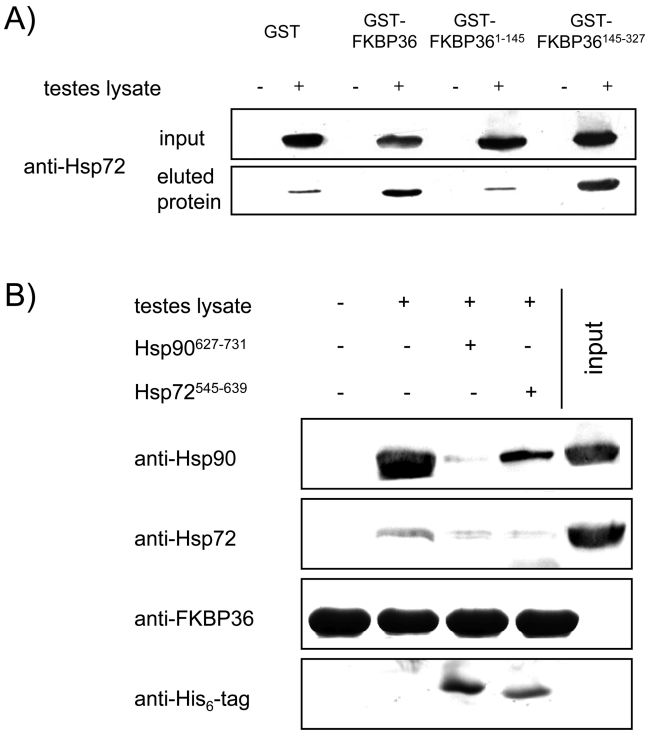

FIGURE 4: FKBP36 TPR domain interacting with the C-terminal Hsp72 domain. (A) Rat testes lysate was incubated with GST−FKBP36 variants or GST-loaded GSH-Sepharose. The input and eluate were analyzed by SDS−PAGE and Western blotting using anti-Hsp72 antibody. (B) Western blot analysis of a competition between Hsp72 and Hsp90 on GST−FKBP36 protein-loaded GSH-Sepharose using anti-Hsp90 and anti-Hsp72 antibodies. The samples were incubated either in the presence or in the absence of Hsp90 C90 or Hsp72 C10. The input and eluate were subjected to SDS−PAGE. The presence of Hsp90 C90 and Hsp72 C10 was analyzed with anti-$(His)_5$ antibodies.

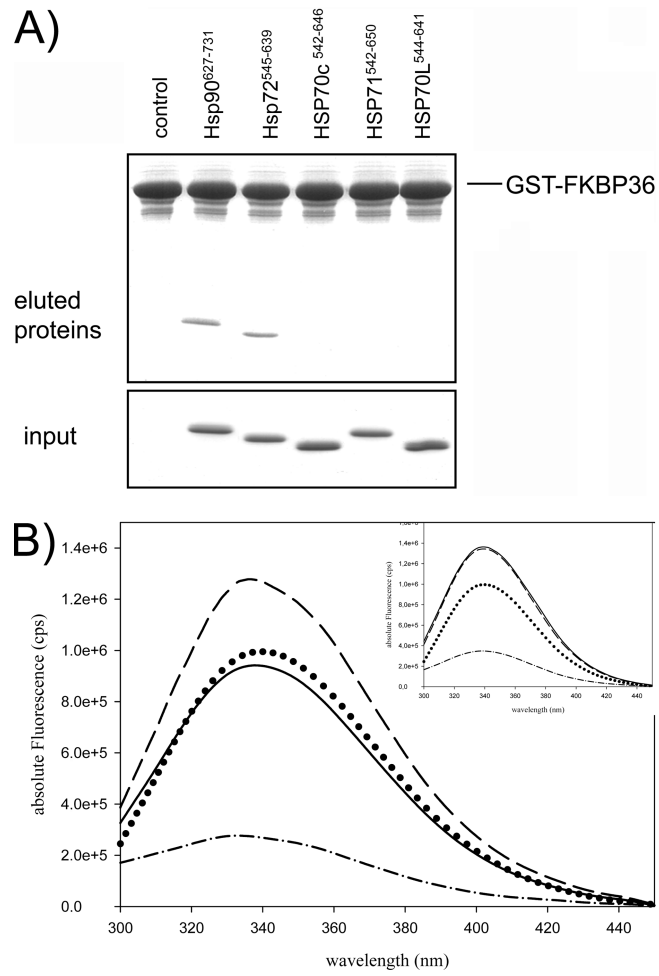

FIGURE 5: FKBP36 discriminates between different Hsp70 proteins. (A) Coomassie-stained SDS−PAGE (15%) of purified C-terminal domains of Hsp90, Hsp72, Hsp71, Hsc70, and Hsp70L incubated with GST−FKBP36 protein-preloaded GSH-Sepharose. The input and eluate were additionally analyzed by Western blotting using anti-$(His)_5$ antibodies (bottom panels). (B) Fluorescence measurements of 1 μM FKBP36 (•••), 1 μM Hsp72 C10 (or Hsp70L C10, inlay) (−•−), and a 1:1 mixture (—) were performed at an excitation wavelength of 280 nm. In addition, the calculated spectrum (− − −) represents the sum of the individual protein spectra, as it should appear when the components do not interact.

both CaM-Ca^{2+}-bound FKBP38 and FKBP36 R81L have comparable activities that are significantly lower than those of constitutively active FKBP family members. FKBP36 may bind to an unknown activating factor or is perhaps phosphorylated at surface residues Tyr^{75} and Thr^{90}, which then may form salt bridges with the Arg^{81} side chain and thus remove the positive charge form the hydrophobic putative active site to activate the PPIase in a manner similar to that of FKBP38.

Functional interactions of the FKBP36 PPIase domain, even without an involvement of the putative active site, mediate the interactions with CHC of the clathrin triskelion. Similar interactions between the PPIase domains of FKBP52 and structural proteins without the participation of the PPIase activities have been reported previously (*15*). In parallel to these complexes, FKBP36 interacts also via its TPR domain with the C-terminal domains of heat shock proteins, which has also been found for FKBP38, FKBP51, FKBP52, and FKBP37.7 (*16–18*). Thus, FKBP36 might mediate the assembly of multiprotein complexes associated with clathrin-coated vesicles in parallel to the role of FKBP52 in steroid hormone receptor complexes (*19*). Within these complexes, FKBP36 might link clathrin and either Hsp72 or Hsp90 to yet unknown client proteins.

However, the interactions among FKBP36, clathrin, and Hsp72 could also be important in another process in spermatocytes: Hsp72 is closely related to Hsc70, which was shown to disassemble the clathrin matrices (*20*). Hsc70 is localized to clathrin-coated vesicles due to interactions with auxillin which, in turn, binds to CHC (*21*). However, auxillin is not present in spermatocytes (*22*), suggesting the involvement of other proteins in the disassembly of clathrin-coated vesicles. Since FKBP36 binds directly to CHC and Hsp72 in testis cells, as demonstrated by the fractionation of rat testes lysate, the resulting clathrin−FKBP36−Hsp72 complexes may have a role in the disassembly of the clathrin coat in the maturation process of this vesicle type.

Our results demonstrate, furthermore, specific binding between FKBP36 and Hsp72. Thus, FKBP36 is the first example of a TPR-containing protein that discriminates between different members of the Hsp70 family. Interestingly, both FKBP36 and Hsp72 play a role in meiosis regulation (*3, 23*). Moreover, the knockout of the genes encoding FKBP36 or Hsp72 individually results in similar phenotypes, with infertile males and perfectly normal females. Thus, FKBP36 and Hsp72 appear to participate in the same process in male meiosis, likely forming FKBP36−Hsp72 complexes. Thus, the testis-specific FKBP36 partici-

pates in several distinct processes in spermatocytes, which depend on its interactions with different cellular partners.

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REFERENCES

1. Meng, X., Lu, X., Morris, C. A., and Keating, M. T. (1998) A novel human gene FKBP6 is deleted in Williams syndrome. *Genomics 52*, 130–137.
2. Tassabehji, M. (2003) Williams-Beuren syndrome: A challenge for genotype-phenotype correlations. *Hum. Mol. Genet. 12* (Special Issue 2), R229–R237.
3. Crackower, M. A., Kolas, N. K., Noguchi, J., Sarao, R., Kikuchi, K., Kaneko, H., Kobayashi, E., Kawai, Y., Kozieradzki, I., Landers, R., Mo, R., Hui, C. C., Nieves, E., Cohen, P. E., Osborne, L. R., Wada, T., Kunieda, T., Moens, P. B., and Penninger, J. M. (2003) Essential role of Fkbp6 in male fertility and homologous chromosome pairing in meiosis. *Science 300*, 1291–1295.
4. Fischer, G., and Aumuller, T. (2003) Regulation of peptide bond cis/trans isomerization by enzyme catalysis and its implication in physiological processes. *Rev. Physiol. Biochem. Pharmacol. 148*, 105–150.
5. Liu, J., Farmer, J. D., Jr., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell 66*, 807–815.
6. Lamb, J. R., Tugendreich, S., and Hieter, P. (1995) Tetratrico peptide repeat interactions: To TPR or not to TPR? *Trends Biochem. Sci. 20*, 257–259.
7. Pratt, W. B., Galigniana, M. D., Harrell, J. M., and DeFranco, D. B. (2004) Role of hsp90 and the hsp90-binding immunophilins in signalling protein movement. *Cell. Signalling 16*, 857–872.
8. Smith, D. F., Baggenstoss, B. A., Marion, T. N., and Rimerman, R. A. (1993) Two FKBP-related proteins are associated with progesterone receptor complexes. *J. Biol. Chem. 268*, 18365–18371.
9. Michnick, S. W., Rosen, M. K., Wandless, T. J., Karplus, M., and Schreiber, S. L. (1991) Solution structure of FKBP, a rotamase enzyme and receptor for FK506 and rapamycin. *Science 252*, 836–839.
10. Kay, J. E. (1996) Structure-function relationships in the FK506-binding protein (FKBP) family of peptidylprolyl cis-trans isomerases. *Biochem. J. 314*, 361–385.
11. 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.
12. Harrison, R. K., and Stein, R. L. (1990) Substrate specificities of the peptidyl prolyl cis-trans isomerase activities of cyclophilin and FK-506 binding protein: Evidence for the existence of a family of distinct enzymes. *Biochemistry 29*, 3813–3816.
13. Daugaard, M., Rohde, M., and Jaattela, M. (2007) The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS Lett. 581*, 3702–3710.
14. Schiene-Fischer, C., Habazettl, J., Schmid, F. X., and Fischer, G. (2002) The hsp70 chaperone DnaK is a secondary amide peptide bond cis-trans isomerase. *Nat. Struct. Biol. 9*, 419–424.
15. Silverstein, A. M., Galigniana, M. D., Kanelakis, K. C., Radanyi, C., Renoir, J. M., and Pratt, W. B. (1999) Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus. *J. Biol. Chem. 274*, 36980–36986.
16. Edlich, F., Erdmann, F., Jarczowski, F., Moutty, M. C., Weiwad, M., and Fischer, G. (2007) The Bcl-2 regulator FKBP38-calmodulin-Ca^{2+} is inhibited by Hsp90. *J. Biol. Chem. 282*, 15341–15348.
17. Pirkl, F., and Buchner, J. (2001) Functional analysis of the Hsp90-associated human peptidyl prolyl cis/trans isomerases FKBP51, FKBP52 and Cyp40. *J. Mol. Biol. 308*, 795–806.
18. Meyer, B. K., and Perdew, G. H. (1999) Characterization of the AhR-hsp90-XAP2 core complex and the role of the immunophilin-related protein XAP2 in AhR stabilization. *Biochemistry 38*, 8907–8917.
19. Davies, T. H., and Sanchez, E. R. (2005) FKBP52. *Int. J. Biochem. Cell Biol. 37*, 42–47.
20. Greene, L. E., and Eisenberg, E. (1990) Dissociation of clathrin from coated vesicles by the uncoating ATPase. *J. Biol. Chem. 265*, 6682–6687.
21. Holstein, S. E., Ungewickell, H., and Ungewickell, E. (1996) Mechanism of clathrin basket dissociation: Separate functions of protein domains of the DnaJ homologue auxilin. *J. Cell Biol. 135*, 925–937.
22. Lemmon, S. K. (2001) Clathrin uncoating: Auxilin comes to life. *Curr. Biol. 11*, R49–R52.
23. Eddy, E. M. (1999) Role of heat shock protein HSP70-2 in spermatogenesis. *Rev. Reprod. 4*, 23–30.
24. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., and Schmid, F. X. (1989) Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature 337*, 476–478.
25. Blobel, G., and Potter, V. R. (1966) Nuclei from rat liver: Isolation method that combines purity with high yield. *Science 154*, 1662–1665.
26. Pearse, B. M., and Robinson, M. S. (1984) Purification and properties of 100-kd proteins from coated vesicles and their reconstitution with clathrin. *EMBO J. 3*, 1951–1957.

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