Kidney Androgen-regulated Protein Interacts with Cyclophilin B and Reduces Cyclosporine A-mediated Toxicity in Proximal Tubule Cells*

Cristina Cebrián‡, Cristina Aresté‡, Antoni Nicolás‡, Pere Olivé‡, Ana Careller§, Jaume Piulats§, and Anna Meseguer¶‡

Published, JBC Papers in Press, April 25, 2001, DOI 10.1074/jbc.M102916200

From the ‡Centre d’Investigacions en Bioquímica i Biologia Molecular, Hospital Universitari Vall d’Hebron, Pg. Vall d’Hebron 119-129, Plta. 14, 08035 Barcelona, Spain and the §Laboratorio de Bioinvestigación, Merck Farma y Química, S.A., 08010 Barcelona, Spain

The gene for kidney androgen-regulated protein (KAP) is the most abundant and specific gene expressed in mouse kidney proximal tubule cells, where it is tightly regulated by steroid and thyroid hormones in different tubule segments. Despite the cell-specific expression, strict regulatory mechanisms, and relative abundance, nothing is known of the function of its encoded protein, which does not exhibit known structural or functional domains, or homologies with other sequences in the data bases. We raised monoclonal antibodies against KAP, which specifically recognize a protein with an apparent molecular mass of 20 kDa in crude kidney homogenates, the distribution and regulation of which parallel that of its mRNA. To gain insight into its function, we performed a yeast two hybrid screen and determined that KAP specifically interacts with cyclophilin B. Furthermore, cyclosporine A (CsA)-treated mice exhibited a significant decrease in KAP levels, and tetracycline-controlled overexpression of KAP in stably transfected proximal tubule cells significantly decreased the toxic effects of CsA. Taken together, these results indicate a functional relationship among KAP-, cyclophilin B-, and CsA-mediated nephrotoxicity and suggest an important role of KAP in renal physiology, providing new data on the molecular mechanisms implied in the toxic effects of CsA.

The kidney androgen-regulated protein (KAP) was identified as an abundant 20,000-dalton protein by in vitro translation of male mouse kidney mRNA (1). Shortly after its identification, it became a useful model for studying kidney-specific hormonal regulation of gene expression. The KAP gene exhibits androgen-dependent and -independent regulatory mechanisms in different segments of proximal tubules (2). Whereas a functional androgen receptor and testosterone are required for expression in cortical S1 and S2 segments of proximal tubules, no androgen is required for expression in the medullar S3 segment (3, 4). Therefore, females, castrated males, and androgen-receptor-deficient Tfm/Y males express the gene in S3 cells exclusively, whereas males and androgen-induced females also express the gene in S1/S2 cells. KAP mRNA expression in S3 cells depends on thyroid hormone (5) and can be further induced by estrogens in females (6). Estrogen-dependent expression of the KAP gene in rat and mouse uteri around delivery has also been reported (7, 8). This complex regulation of KAP is actually more sophisticated because it has also been determined that both thyroid hormone (9) and the growth hormone/insulin-like growth factor-1 (GH/IGF-1) axis (9) cooperate with androgens in promoting KAP gene expression in S1/S2 cells.

KAP constitutes the most abundant and specific gene expressed in proximal renal tubule cells, as shown by serial analysis of gene expression (10), serial analysis of differential expression (11), and expression profiling of active genes (12). Cell specificity and the complex regulatory mechanisms involved in KAP mRNA expression, together with KAP mRNA relative abundance, point to an important role for its encoded protein.

Analyses of both nucleotide and peptide sequences have failed to reveal significant homology with other genes, expressed sequence tags, or proteins or with known structural or functional domains. The absence of known functional domains has greatly reduced experimental approaches to elucidating KAP function and prompted us to perform a yeast two hybrid assay. The present work shows that KAP interacts with the CsA-binding protein CyPB, that CsA administration reduces KAP steady-state levels in mouse kidney, and that KAP overexpression reduces CsA toxicity in cultured proximal tubule cells. Thus, our data indicate a functional relationship among KAP, CyPB, and CsA-mediated toxicity in kidney.

EXPERIMENTAL PROCEDURES

Animals—Six-week-old C57BL/6 and BALB/c mice were purchased from IFFA CREDO (L’Arbresle, France). When required, males were castrated under droperidol and midazolam anesthesia and allowed to recover for 8 days before further treatment. Female, male, and castrated male mice were treated with 3 days with CsA (Sandimmun, Sandoz, Nuremberg, Germany) with intramuscular injection 15 mg/kg/day.

* This work was supported by Grant PM97-0095 from the Ministerio de Educacion y Cultura, Programa Sectorial de Promocion General del Conocimiento. This work has been awarded the In˜igo Alvarez de Toledo prize for the basic research in Nephrology (Edition XII, 2000). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 34-93-4894061; Fax: 34-93-4894064; E-mail: meseguer@hg.vhebron.es.

¶ The abbreviations used are: KAP, kidney androgen-regulated protein; CsA, cyclosporine A; CyP, cyclophilin; GFP, green fluorescent protein; GST, glutathione S-transferase; abKAP, monoclonal antibody against KAP; RT, reverse transcription; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; Tc, tetracycline; BSA, bovine serum albumin; PBS, phosphate-buffered saline; β-gal, β-galactosidase; Oatp1, organic anion transporting polypeptide 1; ER, endoplasmic reticulum; SPDP, N-succinimidyl-3-(2-pyridyldithio) propionate.

2 M. Soler, E. Solé, A. Menoyo, H. Hardy, J. F. Catterall, A. Vandewalle, and A. Meseguer, unpublished results.
Control mice received vehicle alone (95% olive oil, 5% ethanol). After treatment, animals were euthanized by cervical dislocation. Several tissues were collected and immediately frozen in liquid N₂ or embedded in OCT compound and frozen in cold 2-methylbutane.

**RNA Extraction and Northern Blot Analysis**—Total RNA was extracted from each tissue using Trizol reagent (Life Technologies, Inc.) and treated with DNAse and RNase. Aliquots of total RNA (10 μg) were denatured with formaldehyde and separated on 1% agarose/formaldehyde gels and transferred to ZetaProbe membranes (Bio-Rad). Membranes were hybridized at 42 °C overnight with random primed [³²P]dCTP (Amersham Pharmacia Biotech)-labeled cDNA probes, washed following the manufacturer’s instructions, and exposed to X-ray film (Amersham Pharmacia Biotech). Where necessary, membrane intensity was measured by densitometric scanning of the resultant autoradiograph using the Bio-Rad GS700 image densitometer and the Molecular Analyst 1.40 program.

**RT-PCR and Southern Blotting**—RT-PCRs were performed under linear conditions with respect to RNA input and the number of amplification cycles. PCRs were defined as linear for 18–24 cycles. Primers for KAP1 and KAP2 were designed to amplify specific sequences of KAP1 and KAP2 coding for proteins P53 and large T antigen of SV40 were used as a positive control for interaction. Plasmids from these clones were purified and cotransfected with pBD-KAP and with control plasmids in order to confirm the interaction. In this work, the use of β-gal assays was performed as described above. Liquid β-gal assays were performed as previously reported.

**Expression and Purification of Recombinant Proteins**—E. coli (JM109 bacteria) were transformed with constructs expressing GST and GST-KAP. Luria broth (100 ml) containing 50 μg/ml ampicillin was inoculated with 1 ml of bacteria and incubated in an orbital shaker at 37 °C. Bacteria were grown to A₆₀₀ = 0.5, induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside, and shaken for 3 h at 37 °C. The GST-soluble protein was purified by B-PER™ reagent (Pierce). The recombinant GST-KAP aggregates into inclusion bodies. Therefore, the inclusion bodies were solubilized with 6x gdmchexane HCl and refolded with DSB 201 as previously reported (57).

Soluble lysate was conjugated with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) overnight at 4 °C. Beads were washed twice with PBS, and protein concentrations were assessed after electrophoresis on 10% polyacrylamide denaturing gels by Coomassie Blue.

**Immunohistochemistry**—Thin kidney sections (5 μm) were obtained in a cryostat (Bright Instrument Company Ltd., Huntingdon, United Kingdom). Sections were dehydrated in acetone and mounted in DPX mounting medium (Agar Scientific Ltd., Essex, United Kingdom).

**Western Blot Analysis**—Tissues were homogenized by N₂ cavitation in RIPA buffer (DOC 0.5%, Nonidet P-40 1%, SDS 0.1%, and protease inhibitors in 1× PBS). For Western blot analysis, samples were normalized for protein concentration using bovine serum albumin (BSA). Membranes were probed for 2 h. Washes were performed following the membrane manufacturer’s instructions and secondary antibody (horseradish peroxidase-conjugated rabbit anti-mouse, DAKO A/S) diluted 1:5000 and incubated for 1 h at room temperature. After washing, bands were detected using the ECL chemiluminescence detection method (Amersham Pharmacia Biotech) and exposed to Hyperfilm.

**Monoclonal Antibody Characterization**—Antibody isotyping was performed by ELISA using a positive control for interaction. Antibodies were purified from spent culture media with HiTrap protein A columns (Amersham Pharmacia Biotech) using the manufacturer’s instructions. A total of 6 × 10⁵ putative interacting clones were identified by growth in selective media (Leu−, Trp−, His+). From them, 10 clones were positive when screened for β-gal expression. Plasmids from these clones were purified and cotransfected with pBD-KAP and with control plasmids in order to confirm the interaction. The following standards were used as a positive control for interaction.

**Monoclonal Antibody Characterization**—Antibody isotyping was performed by ELISA using a monoclonal antibody-based Ig isotyping kit (PharMingen, San Diego, CA). 388-1D7 was found to be of IgM,k and used as the positive control for interaction. Monoclonal antibodies produced by 377-1B8 and 388-1D7 were used as positive and negative controls, respectively. Hybridomas supernatants were incubated for 2 h, and bound antibodies were detected using the diaminobenzidine substrate solution. Nuclei were stained with hematoxylin-eosin solution, and slides were dehydrated and mounted in DPX mounting medium (Agar Scientific Ltd., Essex, United Kingdom).

**KAP Interacts with CyPB: Implications in Nephrotoxicity**
KAP Interacts with CyPB: Implications in Nephrotoxicity

| Primer name | Orientation | Sequence |
|-------------|-------------|----------|
| CyPB1       | Upper       | 5’-TGA ATT CTC GGA GCG CAA TAT GAA-3’ |
| CyPB2       | Lower       | 5’-TCT CGA GGG CAA AAT TAT CCA CTG-3’ |
| CyPB3       | Upper       | 5’-TGA ATT CTC GGA GCG CAA TAT GAA-3’ |
| CyPB4       | Lower       | 5’-TCT CGA GTC CCC CAG GCT CTC TAC-3’ |
| KAP1        | Upper       | 5’-TAT GTC GAC GCA TGA TGC TTT TCA AGG-3’ |
| KAP2        | Lower       | 5’-TAT GTC GAC GCA TGA TGC TTT TCA AGG-3’ |
| KAP3        | Upper       | 5’-TAT GTC GAC GCA TGA TGC TTT TCA AGG-3’ |

**RESULTS**

**Tissue-specific Distribution of KAP mRNA and Identification of Its Encoded Protein**—Although tissue specificity of the KAP mRNA has been previously reported (17), the more sensitive RT-PCR/Southern technique was used, and distribution of KAP mRNA was performed in a wider panel of tissues. Results shown in Fig. 1A confirm that KAP mRNA is exclusively expressed in the kidney and uterus of pregnant female mice from day 13 of gestation. Although not included in the panel, and confirming previous results (7), we also observed that KAP mRNA becomes undetectable in mouse uterus right after birth.

Computational analysis of the KAP (GenBank™ accession number M22810) deduced peptide sequence defines a protein of 121 amino acids in length, hydrophilic and negatively charged (2). IgG1 monoclonal antibodies against KAP were generated using a male mouse kidney cDNA library in a yeast two hybrid assay. One of the clones was effectively detected by the in vitro translated KAP that migrates with an apparent size of 20 kDa in SDS-PAGE from mouse crude kidney extracts, which was not detectable in liver and lung (Fig. 1B) or in kidney when peptide-specific preabsorbed antibodies were used (not shown). The antibodies were also able to recognize the in vitro translated KAP that migrates with an apparent size of 19 kDa in SDS-PAGE (Fig. 1B, panel 1) and the expected 48-kDa recombinant GST-KAP, heterologously produced in *Escherichia coli* (Fig. 1B, panel 2).

Immunohistochemistry assays in frozen kidney sections identified KAP in epithelial cells of proximal convoluted tubules, which correlates with KAP mRNA site synthesis (Fig. 1C). Antibodies preabsorbed with their corresponding antigenic peptides failed to recognize the KAP target protein in kidney, thereby proving the specificity of the immune reaction (Fig. 1C).

The Kidney Androgen-regulated Protein Resembles Its mRNA Cell Distribution and Androgen Regulation—To test whether the cell distribution and abundance of KAP are also androgen-dependent, Western blot and immunohistochemistry assays were performed in kidneys of intact male, female, and castrated male mice. Results in Fig. 2A demonstrate that the relative amounts of KAP correlate with previously reported relative mRNA levels (2), being much more abundant in males than in females and exhibiting the lowest levels in castrated males. Like its mRNA, KAP is expressed in S3 cells, but it is only expressed in the S1/S2 segments of intact males (Fig. 2B).

The Kidney Androgen-regulated Protein Specifically Interacts with Cyclophilin B (CyPB)—The full-length KAP cDNA was screened against a male mouse kidney cDNA library in a yeast two hybrid assay. One of the clones was effectively...
interacting with KAP to support the permissive growth of yeast cells in selective medium and to produce positive results in β-gal assays (Fig. 3A, section 1). This clone was identified as the mouse peptidyl-prolyl-cis-trans-isomerase B (CyPB, GenBank™ accession number M60456). Positive and negative interacting control proteins were also included (Fig. 3A, sections 2–4, respectively). A GST pull-down assay was performed to further confirm the KAP-CyPB interaction. Sepharose 4B-conjugated GST and GST-KAP fusion proteins were incubated with [35S]methionine-labeled in vitrotranslated CyPB or Oatp1 proteins and extensively washed (four times) in GST wash buffer. Eluted products shown in Fig. 3B demonstrate that the binding capacity of CyPB for GST-KAP depends on the KAP moiety, because GST alone is unable to bind CyPB. Similarly, KAP binds specifically to CyPB because the Oatp1-translated product does not get bound to KAP, thereby demonstrating the specificity of the interaction. When CsA (10 and 25 μM) was added to the interacting proteins, no competitive effect of the immunosuppressor was observed when CyPB was preincubated with CsA or when the immunosuppressor was added to the KAP-CyPB complex (data not shown).

Finally, co-immunoprecipitation assays were performed on kidney crude extracts as an in vivo demonstration of the interaction (Fig. 3C). Although we were able to recover CyPB from the abKAP1 and abKAP2 immunoprecipitated products (Fig. 3C, lanes 1 and 3, respectively), the reverse experiment, i.e. immunoprecipitating with the antibody against CyPB and dem-
KAP Interacts with CyPB: Implications in Nephrotoxicity

KAP 1–321 and KAP 1–437 constructs were able to grow in selective medium, whereas the KAP 1–194 construct was not (Fig. 5B). These results indicate that the CyPB binding capacity exhibited by KAP must be located between base pairs 194 and 321 of the cDNA, i.e., in a region of ~40 amino acids in length, in the middle of the protein. The reverse experiment was also performed and different CyPB overlapping fragments, such as those from base pairs 28–231, 28–515, 231–664, and 515–664 of the cDNA, tested against the full KAP open reading frame. As shown in Fig. 4B, only fragments CyPB 231–664 and CyPB 515–664 were positive. Because CyPB 28–515 was negative, the region encompassing base pairs 551–664, which codes for 37 amino acids of the C-terminal domain, is the one responsible for KAP interaction. These results were further confirmed by the β-gal liquid assays (Fig. 5C).

Effects of CsA Treatment on Kidney KAP Expression: Correlation between the mRNA and the Protein Levels—CsA binds to CyPB and modulates the expression of several genes (19), some of which, but not all, are implicated in the immune response. Because treatment with CsA increases CyPB plasma levels (20) and CyPB has been found to interact with KAP, we aimed to observe the effects of acute CsA treatment on KAP kidney expression under different hormonal conditions.

Female, male, and castrated male mice were treated for 3 days with CsA at 15 mg/kg/day by intramuscular injection and compared with vehicle-treated mice. Results shown in Fig. 5A demonstrate a significant 2.2- and 3.6-fold increase in KAP/glyceraldehyde-3-phosphate dehydrogenase ratios in CsA-treated males and females, respectively, and no effect on KAP mRNA levels in castrated males (Fig. 6A). Western blot analysis performed in crude extracts of counterpart kidneys revealed a significant decrease in KAP in castrated males and females that was not apparent in males (Fig. 6B). The hypothesis that CsA treatment would affect KAP expression preferentially in the S3 segment was proved by immunohistochemistry analysis in frozen kidney sections of CsA-treated and nontreated mice. Fig. 6C demonstrates the lower and almost nonexistent KAP levels in females and castrated males, respectively, and the higher decrease in the protein in S3 cells than in S2 and S1 cells in males.

Production of a Stably Transfected and Tetracycline-controlled KAP Expression System in PKSV-PCT Cells as a Model for Studying the Effects of KAP Expression on CsA-mediated Toxicity—KAP expression vectors were stably transfected in the PKSV-PCT cell line, originally derived from proximal tubules of transgenic mice carrying the large T and little t SV40 antigens, under the control of 5′ regulatory sequences of the t-type pyruvate kinase gene that had proved to conserve the main features of the parental cells from which it was derived (14–16). This cell line was used to express KAP in a controlled and inducible manner using the Tet-Off system from CLONTECH.

Results in Fig. 7A are representative of KAP mRNA levels in pTet-off regulator plasmid stably transfected PKSV-PCT cells (clone 3-26) and in three independent stably expressing tet-regulated pTRE-KAP clones (3-26-37, 3-26-71, and 3-26-310). Endogenous cyclophilin A (CyPA) expression levels were determined as a control for the amount and integrity of mRNA. Whereas results in Fig. 7A indicate that selected clones have the ability to induce KAP mRNA in a tetracycline-dependent controlled manner, those shown in Fig. 7B demonstrate their ability to express KAP in around 3–4 h upon tetracycline removal. Indirect immunofluorescence analysis of expressed KAP shows a cytoplasmic reticular distribution in these cells.

The above-described pTRE-KAP clones were used to assess...
the effects of KAP expression on CsA-mediated toxicity in proximal convoluted tubule cells, using clone 3-26 as a control. Cells were exposed to increasing doses of CsA (from 0 to 50 μg/ml) for a period of 24 h, and culture medium from each situation was assayed for lactate dehydrogenase activity, as a marker of cell death. Results depicted in Fig. 7D for clone 3-26 show the cytotoxicity levels exhibited by these cells at increasing CsA concentrations, which were not modified by tetracycline. Contrarily, in the three independently generated pTRE-KAP clones, tetracycline removal significantly reduced the toxic effects exerted by CsA (see Fig. 7D). These data are representative of results obtained in three independent experiments and clearly demonstrate that KAP expression protein reduces CsA toxicity in proximal tubule cells.

**DISCUSSION**

Since the first description of KAP mRNA in 1979 (1), the identification and function of its encoded protein have remained elusive. This paper reports, for the first time, KAP identification and distribution and makes a direct attempt to gain insight into its function.

The tissue and cell specificity previously reported for KAP mRNA (3–6, 9, 17), were further analyzed by means of the more sensitive RT-PCR/Southern technique in a wider panel of tissues. Kidney specificity and unique expression of KAP in the uterus during a short period of time prior to birth, described earlier by Kasik et al. (7), were definitively assessed in our experiments. No functional data are available to explain the appearance of KAP mRNA in the uterus before delivery, which, for the time being, is associated with the estrogen sensitivity of the gene (6) and the estrogenic peak that occurs at that time in this tissue (7).

Monoclonal antibodies raised against KAP synthetic peptides specifically identified a protein with an apparent molecular mass of 20 kDa in SDS-PAGE that follows the same distribution and androgenic regulation as its encoding mRNA in epithelial cells of proximal convoluted tubules. Although the expected molecular mass for KAP (121 amino acids in length) is around 13 kDa, computational analysis of the KAP-deduced peptide sequence defined a hydrophilic and negatively charged protein (3), which might explain the delay observed in SDS-PAGE.

As our data show KAP to be a highly specific and tightly regulated kidney protein, we hypothesized that it might play an important role in the homeostatic and metabolic events of proximal tubule cells. This notion was further supported by the finding that KAP expression is up-regulated in nephrectomized mouse kidney (21) and that its mRNA levels significantly decrease in a mouse nephrolithiasis model (22). Because no functional or structural domains were identified on the KAP-deduced peptide sequence, we focused our efforts on the identification of proteins able to interact with KAP in vivo, which might in turn be informative of KAP function.

The major finding of the present study was that KAP interacts with cyclophilin B, a member of the immunophilin family that exhibits petidy-l-prolyl-cis-trans-isomerase activity and the ability to bind the potent immunosuppressor CsA (23, 24). The interaction, initially observed by means of the two hybrid assay, was further confirmed by GST pull-down assays and by co-immunoprecipitation of the KAP-CyPB complex from crude kidney extracts. We found these data very interesting because the great clinical benefits of CsA on the improvement of graft survival rates in solid organ transplantation are concomitant with important undesirable nephrotoxic effects (25). The immunosuppressive effects of CsA are known to be related to binding of the immunophilins-CsA complex to the calcium/calmodulin-dependent serine/threonine phosphatase calcineurin, which blocks, in turn, its intrinsic phosphatase activity in vitro (26). As a result, activation and translocation of the nuclear factor of activated T cells are compromised, nuclear factor of activated T cells-supported transcription of the interleukin 2 gene and other cytokine genes is abolished, and T-cell activation is inhibited. FK-506 (tacrolimus), a second T-cell

---

**Fig. 4. Co-location of GFP-KAP and CyPB.** A, PCT cells were transfected with plasmids coding for either GFP alone or GFP-KAP fusion protein. B, immunocytochemistry with antibodies anti-CyPB was performed on GFP-KAP-transfected cells. Nuclei were stained with TO-PRO3. Arrows indicate the reduced amount of CyPB labeling at the plasma membrane of the GFP-KAP-transfected cells.
immunosuppressive drug, binds to a different cellular protein,FKBP12, and the FKBP12-FK 506 complex also binds to and inhibits calcineurin (27).

Whereas the molecular mechanisms involved in immunosuppression are well known, those involved in nephrotoxicity are less understood. CsA exerts its nephrotoxic effects through (i) acute changes in renal hemodynamics (28–32) followed by irreversible striped interstitial fibrosis (33–36), and (ii) cytotoxicity in proximal tubule cells (37–40). In vitro model systems have revealed a site-selective action of the cells of the proximal tubular region of the nephron (41, 42) preferentially in epithelial cells of the S3 segment (43). Recent reports have concluded that CsA exerts a direct toxic effect on proximal cells by reducing DNA synthesis and cell cycle blockade (44), which are coincidental with elevated p53 levels (45). In an experimental model of chronic CsA nephrotoxicity, an increase in apoptotic specific genes has been found, and it has been proposed that increased apoptosis could explain the tubular dropout and loss of cellularity with fibrosis (46).

Apart from these studies, the molecular mechanisms of CsA-induced toxicity in the kidney have not been completely established, nor has it been determined why CsA exerts its deleterious effects on the proximal tubule and preferentially on cells of the S3 segment. The cloning of a third mammalian cyclophilin, CyPC, which in contrast to the widely distributed CyPA and CyPB family members was restricted to the kidney (47) and preferentially expressed in proximal and straight tubules of the kidney (48), suggested the possibility that CyPC could be a mediator of the immuno-suppressive and nephrotoxic actions of CsA, but no functional data have emerged supporting the initial association between CyPC restricted expression and CsA nephrotoxicity.

Identification of the biologically active cyclophilins to promote T cell activation was performed in Jurkat T cells by overexpression of different cyclophilin family members co-transfected with a phosphatase reporter plasmid containing multiple copies of the nuclear factor of activated T cells or nuclear factor-interleukin 2 enhancer (49). Both CyPA and CyPB but not CyPC increased T cell sensitivity to CsA, demonstrating that CyPA and CyPB are the active immunophilins able to mediate the inhibitory effects of CsA in vivo (49). In addition to their ability to bind calcineurin, the subcellular location of each immunophilin was shown to be important for mediating inhibition of signal transduction by CsA. In this regard, deletions of CyPB and CyPC domains, which change their intracellular locations, altered their activities significantly. Although both immunophilins are located in the ER, CyPB is associated with a specialized part of the ER related to calcium activation events, which permits its activity (50). It is interesting to note that the carboxyl-terminal 37 residues of CyPB were able to confer a significant gain in function on CyPC and that the converse construction lowered CyPB activity (49). Both CyPA and CyPB but not CyPC contain the previously characterized location signal, which permits its activity (50). It is interesting to note that the carboxyl-terminal 37 residues of CyPB were able to confer a significant gain in function on CyPC and that the converse construction lowered CyPB activity (49). This region of CyPB contains the previously characterized location signal, which could direct a foreign protein to the specialized CyPB retention site (the calcium-containing ER substructure or calciosome) (50). CyPC exclusion from the calciosome may prevent it from participating in inhibition of T-cell activation. The finding that CyPB interacts with a protein involved in calcium signaling in T cells named CAML (51) reinforces the notion that CyPB participates in inhibition of T-cell activation. The finding that CyPB interacts with a protein involved in calcium signaling in T cells named CAML (51) reinforces the notion that CyPB participates in inhibition of T-cell activation.

Involvement of CyPA and/or CyPB in the nephrotoxic effects of CsA has not been assessed. Although a cyclophilin A knockout mouse has recently been produced, nothing is known on toxicity in these animals, and the question as to which cyclophilin is relevant in nephrotoxicity remains open. Because FK506 exhibits the same toxic effects on kidney as CsA and the only common functional mechanism between both immunosuppressors is calcineurin inhibition, it is accepted that nephrotoxicity will also occur through the inhibition of this calcium calmodulin-dependent phosphatase. Furthermore, chemical modifications of the CsA molecule aiming at eliminating its toxicity while preserving immunosuppressive effects have failed, suggesting that both actions occur through common

---

3 J. Luban, personal communication.
mechanisms (52). Taking into account the wide tissue and cell distribution of cyclophilins, the finding that a kidney-specific protein of the proximal tubule interacts with CyPB (the CyP that shows the highest affinity for CsA (53) and the highest expression levels in kidney (54)) was suggestive of a putative new molecular pathway of CsA-mediated toxicity in this tissue.

**FIG. 6. Effects of CsA treatment on KAP expression.** A, Northern blot assay of mouse kidney (3 μg of RNA poly(A)+/lane). Animals were injected with 15 mg/kg/day of CsA or vehicle (olive oil) for 3 days. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA hybridization in the same filters was used as an internal control for RNA integrity and loading. Ratios between KAP-specific and glyceraldehyde-3-phosphate dehydrogenase-specific signals are indicated. B, Western blot assay of KAP expression in counterpart kidneys of CsA-treated and control intact males (25 μg/lane), females (100 μg/lane), and castrated males (100 μg/ lane). C, immunohistochemistry on kidneys from treated mice. CsA diminishes KAP expression in the S3 cells in male, female, and castrated male mice.

**FIG. 7. KAP overexpression prevents CsA-mediated cytotoxicity in culture proximal tubule cells.** PSVK-PCT cells were transfected with the Tet-Off™ system and assayed for KAP-regulated expression. PCT 3-26 is transfected only with the regulator plasmid pTet-off. PCT 3-26-37, PCT 3-26-71, and PCT 3-26-310 were obtained by stably transfecting PCT 3-26 with the plasmid pTRE-KAP. A, RT-PCR assay with total RNA from selected clones. Cyclophilin A (CypA) was used as an internal control. Cells were seeded in the presence of tetracycline (0.01 μg/ml). After extensive washing, cells were left to grow on media with or without Tc for 24 h; RNA was then obtained, and RT-PCR was performed. B, induction timing for KAP expression. Cells were grown in 0.01 μg/ml of Tc. After complete Tc removal, cultures were assayed for protein expression at different time points (from 0 to 24 h) by Western blot assays with abKAP1. The protein was detectable ~3–4 h post-derepression. C, immunocytochemistry performed on clone PCT 3-26-310 with antibody abKAP1. Staining was present in the cytoplasm, displaying a reticular pattern. Original magnification, × 630. D, cytotoxicity assays. Lactate dehydrogenase activity in culture media from 24 h CsA-treated control Tet-Off™ clone PCT 3-26 and KAP-expressing clones PCT 3-26-37, PCT 3-26-71, and PCT 3-26-310 was measured to evaluate cell death. *, p < 0.001.
the C-terminal domain of CyPB is responsible for interaction. This result was further confirmed in the co-immunoprecipitation assays in which we failed to detect KAP when the immunoprecipitation was performed with an antibody that specifically recognizes the C-terminal end of CyPB. As mentioned previously, this is the domain responsible for CyPB sublocation to the calciosome in the ER and the domain that is highly conserved in CyPBs from different species and not present in other cyclophilins. Interaction of KAP and CyPB was not prevented by CsA, which concur with the fact that domains for CsA binding and peptideyl-prolyl-cis-trans-isomerase (PPIase) activity are in the middle of the protein (55), and that KAP-CyPB interaction takes place at the C-terminal domain.

Apart from being located in the endoplasmic reticulum, CyPB has also been found in the plasma membrane and secreted to the medium (56). Confocal microscopy performed in cultured proximal tubule cells in this study demonstrated that endogenous CyPB in these cells is located in the expected cellular compartments, i.e., the ER and the plasma membrane. Interestingly, we observed that for cells transiently transfected with a GFP-KAP fusion protein expression vector, the overlay of KAP and CyPB shows a perfect co-location for both proteins, with a GFP-KAP fusion protein expression vector, the overlay

Interestingly, we observed that for cells transiently transfected with a GFP-KAP fusion protein expression vector, the overlay of KAP and CyPB shows a perfect co-location for both proteins, with a GFP-KAP fusion protein expression vector, the overlay

Interestingly, we observed that for cells transiently transfected with a GFP-KAP fusion protein expression vector, the overlay of KAP and CyPB shows a perfect co-location for both proteins, with a GFP-KAP fusion protein expression vector, the overlay

Interestingly, we observed that for cells transiently transfected with a GFP-KAP fusion protein expression vector, the overlay of KAP and CyPB shows a perfect co-location for both proteins, with a GFP-KAP fusion protein expression vector, the overlay

Interestingly, we observed that for cells transiently transfected with a GFP-KAP fusion protein expression vector, the overlay of KAP and CyPB shows a perfect co-location for both proteins, with a GFP-KAP fusion protein expression vector, the overlay

Interestingly, we observed that for cells transiently transfected with a GFP-KAP fusion protein expression vector, the overlay of KAP and CyPB shows a perfect co-location for both proteins, with a GFP-KAP fusion protein expression vector, the overlay

Interestingly, we observed that for cells transiently transfected with a GFP-KAP fusion protein expression vector, the overlay of KAP and CyPB shows a perfect co-location for both proteins, with a GFP-KAP fusion protein expression vector, the overlay

Interestingly, we observed that for cells transiently transfected with a GFP-KAP fusion protein expression vector, the overlay of KAP and CyPB shows a perfect co-location for both proteins, with a GFP-KAP fusion protein expression vector, the overlay
KAP Interacts with CyPB: Implications in Nephrotoxicity

44. Healy, E., Dempsey, M., Lally, C., and Ryan, M. P. (1998) *Kidney Int.* **54**, 1955–1966
45. Healy, E., Lally, C., and Ryan, M. P. (1999) *Kidney Int.* **56**, 1254–1257
46. Shihab, F. S., Andoh, T. F., Tanner, A. M., Hong, Y., and Bennett, W. M. (1999) *Kidney Int.* **56**, 2147–2159
47. Friedman, J., and Weissman, I. (1991) *Cell* **68**, 799–806
48. Otsuka, M., Terada, Y., Yang, T., Nonoguchi, H., Tomita, K., and Marumo, F. (1994) *Kidney Int.* **45**, 1340–1345
49. Bram, R. J., Hung, D. T., Martin, P. K., Schreiber, S. L., and Crabtree, G. R. (1993) *Mol. Cell. Biol.* **13**, 4760–4769
50. Arber, S., Krause, K. H., and Caroni, P. (1992) *J. Cell Biol.* **116**, 113–125
51. Bram, R. J., and Crabtree, G. R. (1994) *Nature* **371**, 355–358
52. Sigal, N. H., Dumont, F., Durette, P., Siekirk, J. J., Peterson, L., Rich, D. H., Dunlap, B. E., Staruch, M. J., Melino, M. R., and Koprak, S. L. (1991) *J. Exp. Med.* **173**, 619–628
53. Schneider, H., Charara, N., Schmitz, R., Wehrli, S., Mikol, V., Zurini, M. G. M., Quesniaux, V. F. J., and Movva, N. R. (1994) *Biochemistry* **33**, 8218–8224
54. Kainer, D. E., and Doris, P. A. (2000) *Hypertension* **35**, 958–964
55. Mikol, V., Kallen, J., and Walkinshaw, M. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 5183–5186
56. Price, E. R., Jin, M., Lim, D., Pati, S., Walsh, C. T., and McKeen, F. D. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 3931–3935
57. Goldberg, M. E., Expert-Bezancon, N., Vuillard, L., and Rabilloud, T. (1996) *Fold. Des.* **1**, 21–27
Kidney Androgen-regulated Protein Interacts with Cyclophilin B and Reduces Cyclosporine A-mediated Toxicity in Proximal Tubule Cells
Cristina Cebrián, Cristina Aresté, Antoni Nicolás, Pere Olivé, Ana Carceller, Jaume Piulats and Anna Meseguer

J. Biol. Chem. 2001, 276:29410-29419.
doi: 10.1074/jbc.M102916200 originally published online April 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102916200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 57 references, 13 of which can be accessed free at http://www.jbc.org/content/276/31/29410.full.html#ref-list-1