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Kidney International, Vol. 57 (2000), pp. 1590–1598

Association of cyclophilin A with renal brush border membranes: Redistribution by cyclosporine A

Michel Demeule, Alain Laplante, Arash Sepehr-Araé, Gérard M. Murphy, Roland M. Wenger, and Richard Béliveau

Laboratoire de Médecine Moléculaire, Université du Québec à Montréal-Hôpital Ste.-Justine, Montréal, and Novartis Pharmaceuticals Canada Inc., Dorval, Canada; and Novartis Pharmaceuticals Switzerland Inc., Basel, Switzerland

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Background. Administration of the immunosuppressive agent cyclosporine A (CsA) is associated with nephrotoxicity. The main target for CsA, cyclophilin A (CypA), was found in high levels in epithelial cells of renal proximal tubules. In the present study, CypA was immunodetected and characterized following CsA treatment in subcellular fractions of renal cortex.

Method. The renal content and distribution of CypA was evaluated in untreated rats and in rats treated with a subcutaneous injection of CsA (10 mg · kg^{-1} · day^{-1}) for 10 days.

Results. In untreated rats, membrane-bound CypA represents 0.25% of total brush border membrane (BBM) proteins, similar to the proportion found in the soluble fraction. High ionic strength treatment was unable to extract CypA from BBMs, whereas alkaline treatment (Na_2CO_2, pH 11) and detergent 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfate (CHAPS) released it from BBMs. These results indicate that CypA is associated with renal BBMs, and that hydrophobic interactions are involved in this association. The CypA distribution was strongly modified in both BBMs and the soluble fraction after CsA treatment, but its affinity for CsA estimated by photoaffinity labeling was unaffected. The CypA expression level decreased by 45% in BBMs, while it increased by 33% in the soluble fraction, compared with control rats. CypA remained associated with the membranes following in vitro incubation of renal BBMs with CsA. However, incubation of CypA with one of its substrates released CypA from renal BBMs.

Conclusions. These experiments suggest that renal BBMs contain a significant amount of CypA and chronic exposure to CsA, and acute exposure to one of CypA substrates may modify its subcellular distribution.

Cyclophilin A (CypA) has been identified as the main target for the immunosuppressive agent cyclosporine A (CsA) [1–4]. This protein was shown to be abundant in cytosolic extract from lymphocytes and to possess high affinity for CsA [1]. Following the identification of CypA, other members of the cyclophilins were cloned and characterized. These cyclophilins (CypB, CypC, CypD, and Cyp40) were found to be less abundant and located not only in cytosol, but also in membranes and subcellular organelles because of the presence of hydrophobic N-terminal signal as well as C-terminal extensions [5–8]. More recently, Cyp40 and CypA were shown to be a part of a cytosolic heat-shock protein-immunophilin chaperone complex that also includes caveolin, heat-shock protein 56, and cholesterol [9]. CsA binds to this conserved family of abundant proteins [10, 11] that are expressed in various intracellular compartments in most, if not all, organisms [1, 12–14].

The cyclophilins possess a peptidyl-prolyl *cis-trans* isomerase activity (PPIase) and are thought to be involved in protein folding via *cis-trans* isomerization of proline residues [12, 13]. This PPIase activity suggests that CypA may act as a molecular chaperone, facilitating protein folding and protein trafficking [12, 15]. CsA binds tightly to CypA and inhibits its PPIase activity. However, the inhibition of the PPIase activity by CsA and many of CsA analogues does not correlate with their immunosuppression activities [3, 16]. Immunosuppression was found to be dependent on inhibition of calcineurin, a Ca^{2+}-calmodulin–dependent phosphatase [17]. For its immunosuppressive activity, the exposed portion of CsA in the CsA-cyclophilin complex binds to calcineurin. Formation of this complex inhibits the serine-threonine phosphatase activity of calcineurin [10], thus blocking all subsequent steps in the immunostimulatory signal transduction pathway of T cells [18].

Cyclophilin A is widely distributed in almost all tissues in prokaryotes and eukaryotes. In humans, CypA was found in all organs, and concentrations were between 1 and 3 μg/mg protein [19]. Immunostaining at the cellular level indicated not only cytoplasmic but also nuclear localization of CypA [20]. More particularly, relatively

Key words: nephrotoxicity, immunosuppression, cyclophilins, protein folding, renal cortex.

Received for publication May 13, 1999
and in revised form October 7, 1999
Accepted for publication November 1, 1999

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high levels of CypA were found in the kidney [19, 20]. A distinct regional distribution of CypA was observed in this tissue, where proximal tubular epithelial cells were reported to contain distinctly more CypA than the other structures and glomeruli. The mRNA encoding CypC, another member of the cyclophilin family, was also shown to be expressed in the kidney [21]. In a previous study, the nephrotoxicity effects of CsA were proposed to be mediated through the binding to renal CypA, which caused the inhibition of calcineurin phosphatase activity [22]. In the present study, we report an abundant occurrence of cyclophilin in both soluble fraction and brush border membranes (BBMs) from renal cortex. We have characterized the effects of various agents on the association of CypA with BBMs. We analyzed the photoaffinity labeling of CypA associated with BBMs by diazirine-CsA (SDZ 212-122) and compared it with the one obtained for CypA in soluble fraction and human recombinant CypA. To our knowledge, this is the first report demonstrating an association of CypA with renal BBMs that could be modulated by a CypA substrate (Suc-Ala-Ala-Pro-Phen-pNa peptide) and CsA.

METHODS

Materials

Sprague-Dawley male rats (300 to 350 g) were purchased from Charles River (St.-Constant, Quebec, Canada). The Mini-Protean II apparatus and electrophoresis reagents were from Bio-Rad (Mississauga, Ontario, Canada). Polyvinylidene difluoride (PVDF) membranes and Milliblot-Graphite electroblotter I were from Millipore (Mississauga, Ontario, Canada). Antirabbit, antimouse IgG horseradish peroxidase-linked whole antibody and enhanced chemiluminescence (ECL) reagents were bought from Amersham (Oakville, Ontario, Canada). The polyclonal antibody (pAb) directed against CypA was purchase from BioMol (Plymouth Meeting, PA, USA). The CsA analogue (SDZ 212-122), the parent drug CsA, and the monoclonal antibody (mAb) against CsA were kindly provided by Dr. R.M. Wenger (Novartis, Basle, Switzerland).

Preparation of brush border membranes and soluble fractions

Renal BBMs were prepared by a $MgCl_2$ precipitation method as described previously [23]. The final pellet containing purified BBMs was resuspended in 300 mmol/L mannitol, 20 mmol/L HEPES/Tris, pH 7.5, and was stored at −80°C. The supernatants obtained at the time of BBM precipitation were centrifuged at 100,000 × *g* for 60 minutes to prepare the soluble fractions.

Preparation of nucleus, mitochondria, microsomes, and intracellular membranes

Renal cortex (10 mg/g) was homogenized in buffer containing 2.4 mol/L sucrose and 3.3 mmol/L $CaCl_2$. The homogenate was filtered through a cheese cloth, and the filtrate was centrifuged at 40,000 × *g* for one hour. The pellet was resuspended in 1 mol/L sucrose and 1 mol/L $CaCl_2$ and was centrifuged at 3000 × *g* for five minutes. Then the final pellet was resuspended in 250 mmol/L sucrose and 1 mmol/L $CaCl_2$. For mitochondria and microsome preparations, renal cortex (10 mL/g) was homogenized in 250 mmol/L sucrose, 5 mmol/L Tris/HCl, pH 7.5, and 1 mmol/L ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 600 × *g* for 10 minutes, and the supernatant was centrifuged at 8500 × *g* for 10 minutes. This pellet, washed twice, constituted the enriched mitochondria fraction. The supernatant was centrifuged at 40,000 × *g* for 20 minutes. The supernatant obtained was then centrifuged at 100,000 × *g* for one hour. The final pellet corresponded to the enriched microsomes fractions. For the preparation of intracellular membranes (IMs), kidney homogenate was centrifuged at 40,000 × *g* for 20 minutes to remove unbroken cells, nuclei, and plasma membranes. The supernatant was then centrifuged at 100,000 × *g* for one hour at 4°C to obtain the IMs. The various membrane preparations were resuspended in 50 mmol/L mannitol, 5 mmol/L HEPES/Tris, pH 7.5, and were stored at − 0°C.

Cyclosporine A treatment

Male Sprague-Dawley rats were treated with subcutaneous injections of CsA. For all treatments, each group of animals comprised three rats. CsA was used in physiological buffer containing 147 mmol/L NaCl, 4 mmol/L KCl, 3 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 5 mmol/L glucose, and 15 mmol/L HEPES/Tris, pH 7.4, with 15% (wt/vol) Cremophor EL (CrEL) and 3% (wt/vol) ethanol. The control group received the CsA vehicle (physiological buffer, CrEL, and ethanol), and the treated group was injected daily with CsA (10 $mg \cdot kg^{-1} \cdot day^{-1}$) for 10 days. Rats were sacrificed 24 hours after the last injection. Kidneys from individual animals were pooled, and renal BBMs and soluble fraction were prepared as mentioned previously.

Detection of cyclophilin A by Western blot analysis

Brush border membranes or soluble fraction proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% acrylamide gels according to the method of Laemmli, with a Mini-Protean II apparatus [24]. The proteins were transferred electrophoretically onto a 0.45 μm pore size PVDF membrane with a semidry electroblotter apparatus. The transfer was carried out at 1 mA/cm^2 for 90 minutes with 96

mmol/L glycine, 10 mmol/L Tris, and 10% methanol. PVDF membranes were incubated for 120 minutes at 25°C in Tris-buffered saline (50 mmol/L Tris and 150 mmol/L NaCl, pH 7.0) containing 0.1% Tween-20 (TBS-T) and the anti-CypA pAb (2.5 μg/mL). The membranes were washed three times for 15 minutes in TBS-T and were incubated for 60 minutes with horseradish peroxidase-conjugated antirabbit IgG diluted 1/1000 in TBS-T containing 5% powdered milk. The membranes were finally washed three times in TBS-T for 15 minutes, and the immune complex was revealed with ECL reagents. Protein concentrations were estimated with the Bradford assay [25].

Photolabeling of cyclophilin A with diazirine-cyclosporine A analogue

Brush border membranes or soluble fraction proteins (40 μg) were incubated with diazirine-CsA in 10 mmol/L Tris/HCl, pH 7.0, and protease inhibitors (2 μg/mL aprotinin, 10 μg/mL pepstatin A, and 100 μg/mL bacitracin) for 60 minutes at 25°C, and then were irradiated under a Spectroline ultraviolet lamp (Fisher Scientific, Montreal, Quebec, Canada) at 254 nm on ice. Sample buffer was added to the suspensions, and proteins were resolved by SDS-PAGE on 12.5% acrylamide gels according to the method of Laemmli [24] with a Mini-Protean II apparatus. The proteins were transferred electrophoretically onto a 0.45 μm pore size PVDF membrane with a semi-dry electroblotter apparatus. The transfer was carried out at 1 mA/cm^2 for 90 minutes with 96 mmol/L glycine, 10 mmol/L Tris, and 10% methanol. PVDF membranes were incubated for 120 minutes at 25°C in Tris-buffered saline (50 mmol/L Tris and 150 mmol/L NaCl, pH 7.0) containing 0.2% Tween-20 (TBS-T) and the anti-CsA mAb (2.5 μg/mL). The membranes were washed three times for 15 minutes in TBS-T and incubated for 60 minutes with horseradish peroxidase-conjugated anti-mouse IgG diluted 1/1000 in TBS-T containing 5% powdered milk. The membranes were finally washed three times in TBS-T for 15 minutes, and the immune complex was revealed with ECL reagents.

Densitometric analysis

The intensity of the bands obtained from Western blot analysis and from the photolabeling experiments was estimated with a Personal densitometer SI (Molecular Dynamics, Sunnyvale, CA, USA).

RESULTS

Immunodetection of cyclophilin A in subcellular extracts from renal cortex

Subcellular extracts (soluble fraction, BBM, nucleus, mitochondria, microsomes) were isolated from rat renal cortex. The CypA in these subcellular extracts (10 μg of protein) was detected by Western blot analysis using a pAb directed against CypA (Fig. 1A). The content of this 18 kD protein was higher in the homogenate, followed by BBM, soluble fraction, nucleus, mitochondria, and microsomes. CypA was neither enriched in soluble fraction or BBM, while the enrichment of alkaline phosphatase in these membranes was around 10- to 12-fold over the homogenate. This may be explained by the fact that CypA was not restricted to one of these two cellular compartments. The CypA in soluble fraction and BBM was immunodetected and compared with a standard curve established with different amounts of human recombinant CypA. According to this calibration curve, the estimated CypA concentration was around 2.5 μg/mg in homogenate and BBM, 2 μg/mg in soluble fraction, and 0.5 μg/mg in nucleus, mitochondria, and microsomes.

In addition, the detection of prenylated protein methyltransferase (PPMT), a 33 kD protein specifically localized in the endoplasmic reticulum [26], was also performed using 40 μg of protein from untreated soluble fraction, IMs, and renal BBMs (Fig. 1B). PPMT was detected only in IMs, suggesting that the presence of CypA in BBM is not due to contamination by other IMs. This is in agreement with a previous study in which we showed that BBMs are not enriched in various IM markers [27].

Cyclophilin A was also immunodetected after polyacrylamide gel electrophoresis performed under reducing and nonreducing conditions with heated and nonheated samples (Fig. 1C). These procedures are usually used to reduce disulfide bonds and to minimize proteolytic cleavage by endogenous proteases. The reducing agent, β-mercaptoethanol, in the presence of SDS permits the dissociation of proteins with disulfide cross-link into their subunits. When this reducing agent was added to both renal BBMs and the soluble fraction samples, CypA was detected as a 18 kD protein. In the soluble fraction, other bands at 60, 40, 38, and 30 kD were also revealed by the pAb directed against CypA. Boiling the samples had no effect on CypA detection in both fractions. However, under nonreducing conditions (in absence of β-mercaptoethanol), the immunodetection profile of CypA was strongly affected. In these conditions, no immunoreactive band was revealed at 18 kD, and only one strong band with a high molecular weight (>200 kD) was observed in the soluble fraction. These results indicate that electrophoresis under reducing conditions is essential for CypA detection by Western blot.

Effect of cyclosporine A treatment on renal cyclophilin A content, and cyclophilin A's affinity for cyclosporine A

Because CypA is a target for the immunosuppressive agent CsA, rats were treated with this drug to determine whether it may induce a release of CypA associated with

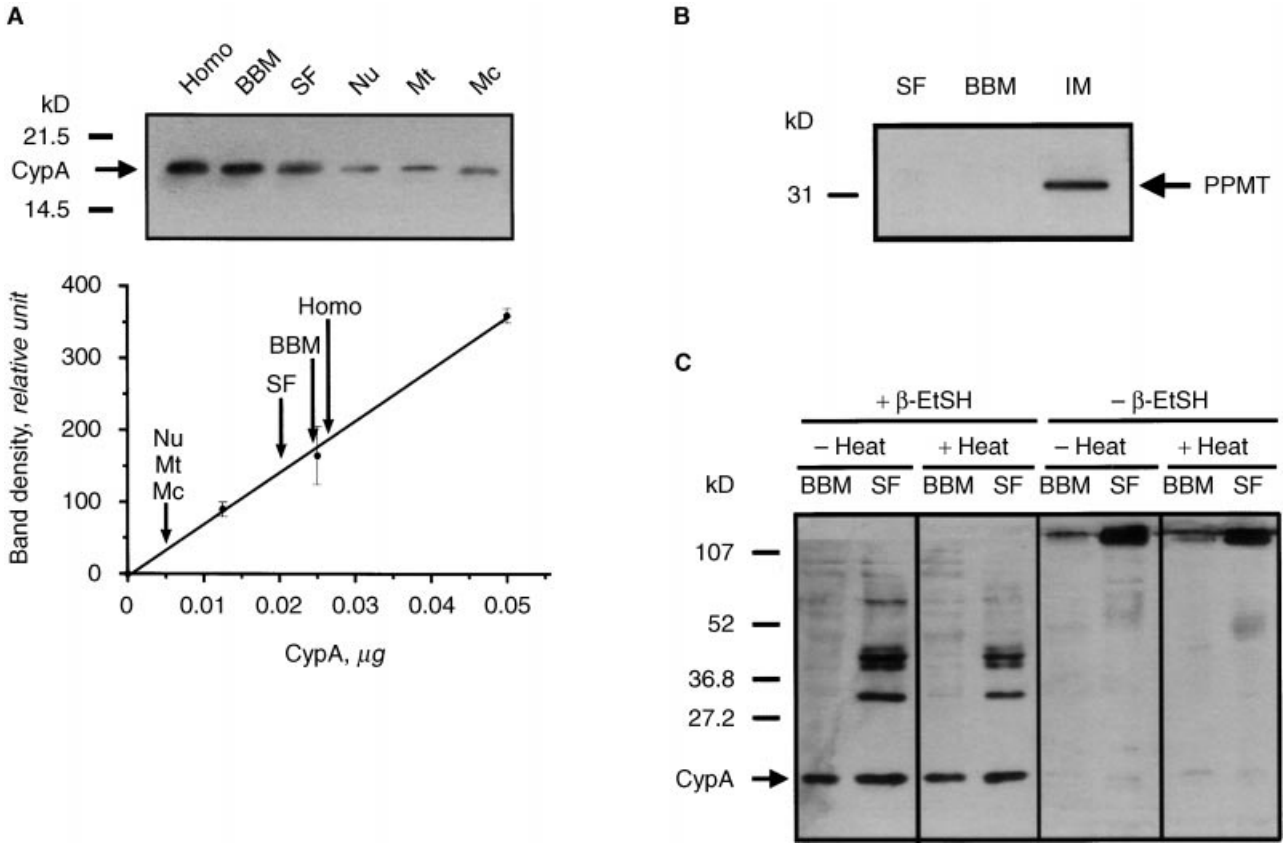

Fig. 1. Immunodetection of cyclophilin A (CypA). (*A*) Subcellular fractions of renal cortex were isolated as described in the **Methods** section. Equal amounts of proteins (10 μg) from renal cortex homogenate (H), brush border membranes (BBMs), soluble fraction (SF), nucleus (Nu), mitochondria (Mt), and microsomes (Mc) were loaded onto 15 wells of a 12.5% acrylamide gel and separated by SDS-PAGE. CypA was detected by Western blot analysis using a polyclonal antibody (pAb) directed against CypA and enhanced chemiluminescence (ECL) reagents. A standard curve using recombinant CypA (0 to 0.05 μg) was also performed, and the densities corresponding to the levels of CypA in the various subcellular fractions are indicated by arrows. (*B*) As a control, phenylated protein methyltransferase (PPMT) was immunodetected in 40 μg of protein from renal SF, BBM, and intracellular membranes (IMs) using a pAb developed in our laboratory that is directed against the amino acid residues 207 to 218 of PPMT from *Xenopus laevis.* (*C*) CypA was immunodetected in 20 μg of protein from renal BBM and SF following eletrophoresis under reducing and nonreducing conditions using a 10-well polyacrylamide gel. Leammli sample buffer with or without β-mercaptoethanol (β-EtOH) was used, and samples were boiled (+ heat) or not boiled (− heat) for five minutes prior to SDS-PAGE. One representative experiment is shown ($N = 3$).

BBM. Rats received subcutaneous injections of CsA (10 $mg \cdot kg^{-1} \cdot day^{-1}$) for 10 days. Soluble fraction and BBMs were purified from the kidney cortex of the different rat groups 24 hours after the last injection. The enrichment values of γ-glutamyltranspeptidase activity for all BBM preparations were similar for the control (8.3 ± 0.7) and treated (8.7 ± 0.6) groups. In the present study, the CypA content and affinity for CsA were evaluated after this CsA treatment. The CypA content after CsA treatment was evaluated in renal homogenate and both soluble fraction and renal BBM (Fig. 2A). As controls, RhoGDI, a cytosolic regulatory protein of Rho, and β-actin were also detected in the same samples by Western blot. RhoGDI was present in renal homogenate and soluble fraction, which is in agreement with its cytosolic localization, whereas β-actin was mainly detected in renal BBM. The content of both RhoGDI and β-actin, as well as other membrane-bound proteins of the small G-protein family such as Ras, Rho A and Rho B, was unchanged after CsA treatment in renal BBM or soluble fraction (data not shown). These results indicate that an equal amount of proteins was applied to the Western blots, and the results suggest that the changes in the CypA content in BBM and soluble fraction are not caused by a physical entrapment of soluble CypA in renal BBM. The levels of CypA, β-actin, and RhoGDI were also evaluated in samples isolated from rats for four days after a single administration of the nephrotoxic agent cisplatin at 5 mg/kg. This dose of cisplatin corresponds to chemotherapeutic levels known to induce mild renal failure in rats [28–30]. We chose day 4 for collecting renal samples because it has been reported that renal insufficiency and functional alterations caused by cisplatin are maximal on days 3 to 5 after treatment with this agent. The levels of CypA were unaffected in both soluble fraction and renal BBM by cisplatin, suggesting that the redistribution of CypA is specific to CsA and that the molecular events that lead to the nephrotoxicity

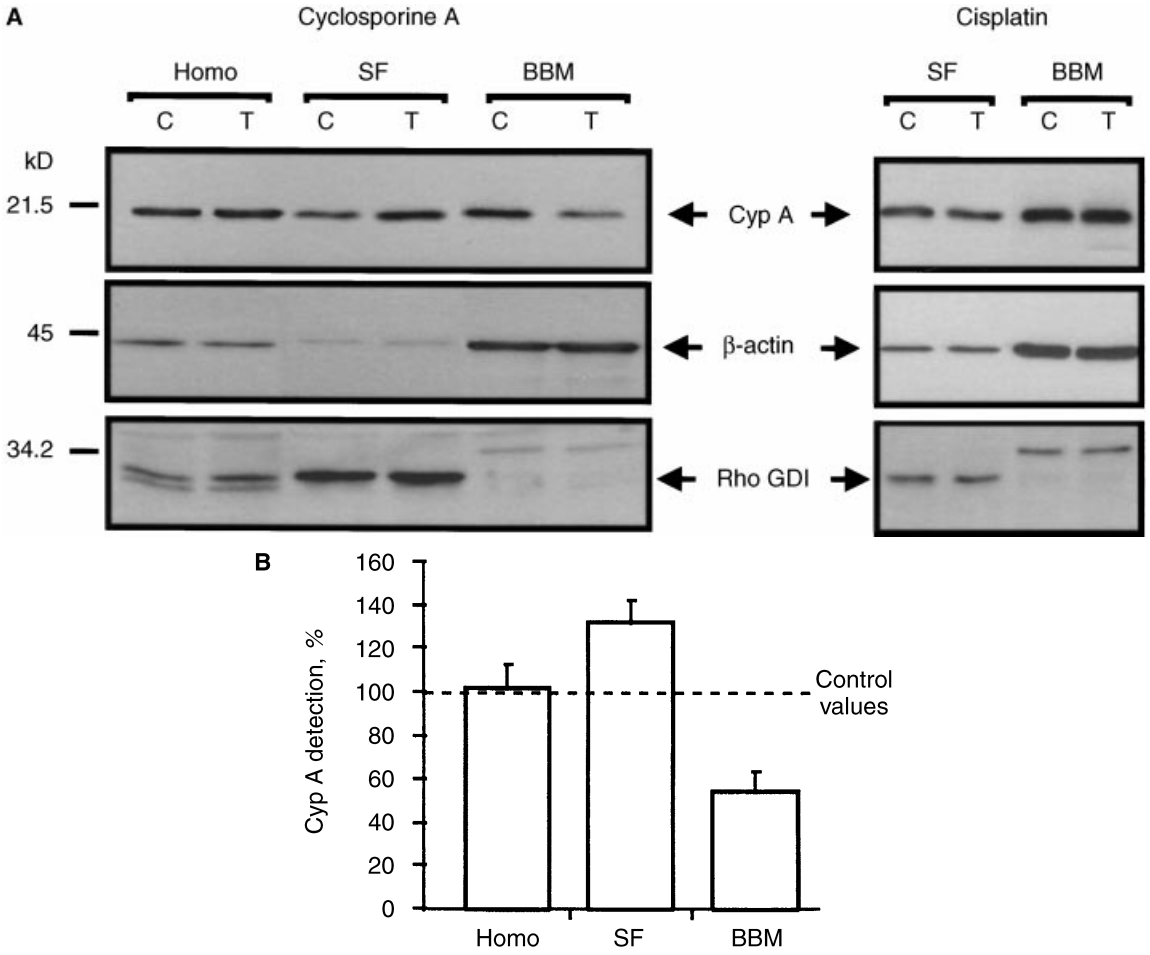

Fig. 2. CypA content in renal BBM and soluble fraction after CsA treatment. (*A*) For cyclophilin A (CypA), equal amounts of proteins (20 μg) from renal homogenate (Homo), soluble fraction (SF), and brush border membrane (BBM) isolated from control (C) and treated (T) rats were resolved by SDS gel electrophoresis with a 12.5% polyacrylamide gel. Immunoblots were performed with a pAb against CypA, as described in the **Methods** section. As a control, RhoGDI and β-actin were immunodetected in the same samples using a pAb as previously described [34]. The levels of CypA, β-actin, and RhoGDI were evaluated also in samples (20 μg of proteins) isolated from control rats and rats treated with a single subcutaneous injection of cisplatin (5 mg/kg) as previously performed [30]. (*B*) The band corresponding to CypA was scanned by laser densitometry, and the content of CypA in renal fractions from CsA-treated rats was expressed as a percentage of CypA present in corresponding fractions from control rats. One representative experiment is shown ($N = 3$ different CsA treatments or cisplatin treatments).

of both drugs are different. Data obtained from three different CsA-treatments indicated that CypA levels increased by 33 ± 9% in the soluble fraction, whereas the content of CypA in renal BBM decreased by 45 ± 10%, suggesting a release of CypA from BBM (Fig. 2B).

Photolabeling of CypA with diazirine-CsA (SDZ 212-122) in both BBM and soluble fractions from renal cortex of control and treated rats was performed to determine whether CypA from treated rats retained its ability to interact with CsA (Fig. 3). Photolabeling of CypA was performed by incubating soluble fraction and renal BBM proteins with diazirine-CsA (1 μmol/L) and increasing concentrations of CsA followed by irradiation. CsA bound to CypA was detected by Western blot analysis using a mAb directed against CsA. The CsA/CypA complex migrated as a 19 kD protein. This complex was also immunodetected using a pAb directed against CypA (data not shown). In the absence of CsA, densitometric analysis from three different CsA treatments indicated that CypA photolabeling in the BBM isolated from rats treated with CsA was reduced by 49 ± 10% (Fig. 3A). This reduction in the photoaffinity labeling of CypA by diazirine-CsA was similar to the decrease in CypA content associated with these BBMs (45%). In contrast, the photolabeling of CypA in the soluble fraction isolated from treated rats evaluated in absence of CsA was reduced by 37 ± 10%, while the CypA content increased by 33 ± 9% (Fig. 3C).

In BBM samples, the photolabeling of the CsA/CypA complex decreased in the presence of increasing concentrations of CsA (Fig. 3B). The concentrations of CsA that reduced the photolabeling of CypA by 50% were identical (0.24 ± 0.02 and 0.23 ± 0.01 μmol/L) for BBM isolated from control or treated rats. The photolabeling

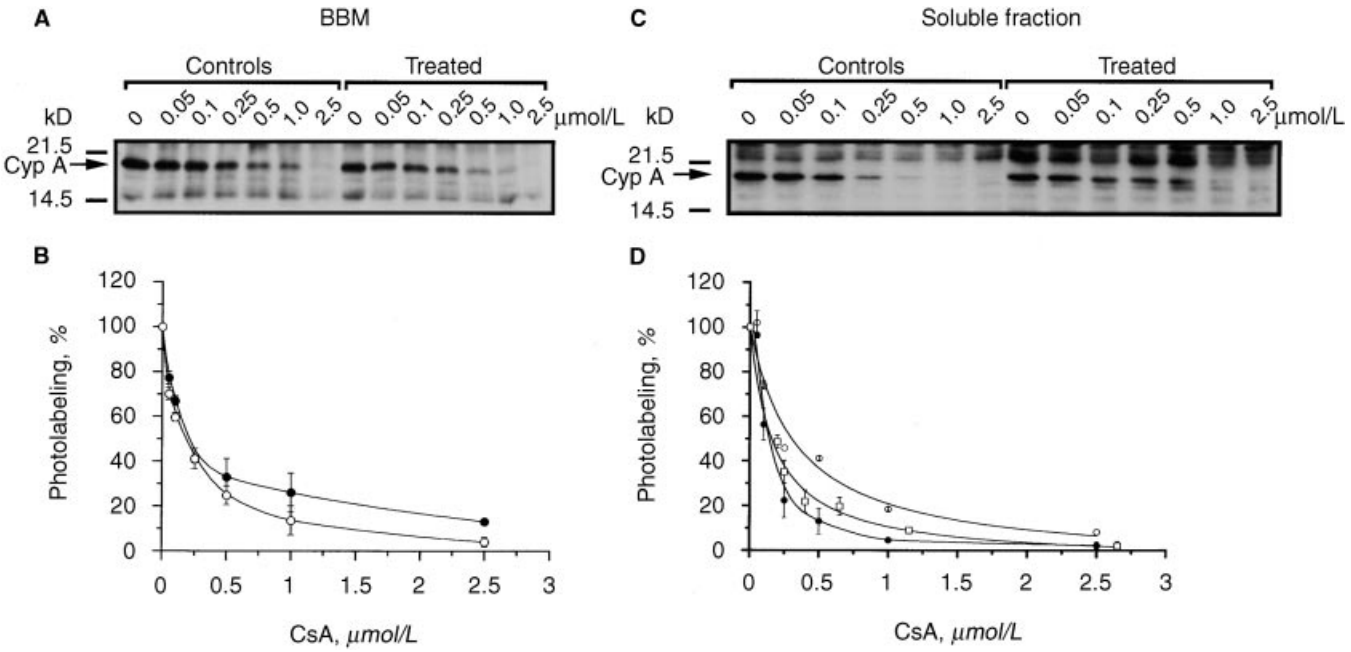

Fig. 3. Photolabeling of CypA in the presence of increasing concentrations of CsA. Photolabeling of CypA in renal BBM (*A*) and soluble fraction (*C*) from both control and treated rats was performed. Equal amounts of proteins (40 μg) were incubated with diazirine-CsA (1 μmol/L) and increasing concentrations of CsA (0 to 2.5 μmol/L) for 60 minutes at 25°C. After irradiation, CsA bound to CypA was detected by Western blot analysis using a mAb directed against CsA, as described in the **Methods** section. The density of the photolabeled CypA complex in renal BBM (*B*) from control (●) and treated (○) rats was expressed as a function of the CsA concentration. (*D*) The density of the photolabeled CypA complex in soluble from control (●) and treated (○) rats was also expressed as a function of the CsA concentration. When the remaining concentration of CsA was taken into consideration, we obtained a new corrected curve (□). Values represent means ± SEM obtained following two different CsA treatments.

of CypA by diazirine-CsA was different in the soluble fraction from CsA-treated rats compared with that from control animals (Fig. 3D). The CsA concentration needed to reduce by 50% the photolabeling of CypA in soluble fractions isolated from treated rats was 0.34 ± 0.04 μmol/L compared with 0.15 ± 0.03 μmol/L for soluble fractions from control rats. To verify whether the difference observed between these two values could be related to the presence of CsA that remains in the soluble fraction of treated rats, the concentration of CsA was estimated by high-performance liquid chromatography. The CsA concentration determined by high-performance liquid chromatography (HPLC) in the soluble fraction after a 10-day treatment was around 1280 ng/L (approximately 1 μmol/L). This concentration of CsA in the soluble fraction from treated rats corresponds to a final concentration of 0.15 to 0.2 μmol/L in the photolabeling assay. This concentration of CsA reduced by 50% the photolabeling of CypA in the soluble fraction from the control group. Thus, the density obtained for the CsA-treated soluble fraction measured in absence of exogenous CsA (0 μmol/L) was corrected by a factor of 2, and each CsA concentration was also corrected for remaining CsA. These corrected density values (%) and CsA concentrations were plotted (Fig. 3D). From this corrected curve, the estimated CsA concentration that displaced by 50% the CypA photolabeling by diazirine-CsA in soluble fractions from control and treated rats was similar at 0.15 ± 0.04 vs. 0.19 ± 0.03 μmol/L, respectively. The endogenous CsA remaining in the soluble fraction after CsA administration thus explained the decrease of CypA photolabeling observed for this fraction in absence of exogenous CsA (0 μmol/L) in Fig. 3C.

Extraction of cyclophilin A from renal brush border membranes

Different agents and conditions were tested to characterize the association of CypA with BBMs (Fig. 4). BBM was incubated in homogenate buffer (low osmotic medium) and in 1 mol/L NaCl, 0.5% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane-sulfate (CHAPS), or 0.2 mol/L Na_2CO_3, pH 11 (Fig. 4A). The suspensions were then centrifuged, and the CypA content was evaluated by Western blots of pellets and supernatants. After the incubation in low osmotic conditions or in high ionic strength, the major portion of CypA was detected in the pellet, suggesting that CypA remained associated with renal BBM. In contrast, the CypA after the incubations with CHAPS or Na_2CO_3, pH 11, was mainly found in the supernatant. These results also suggest that there are at least two populations of CypA in BBM, one that is strongly associated with the membranes and another that could be more easily extracted from the membranes.

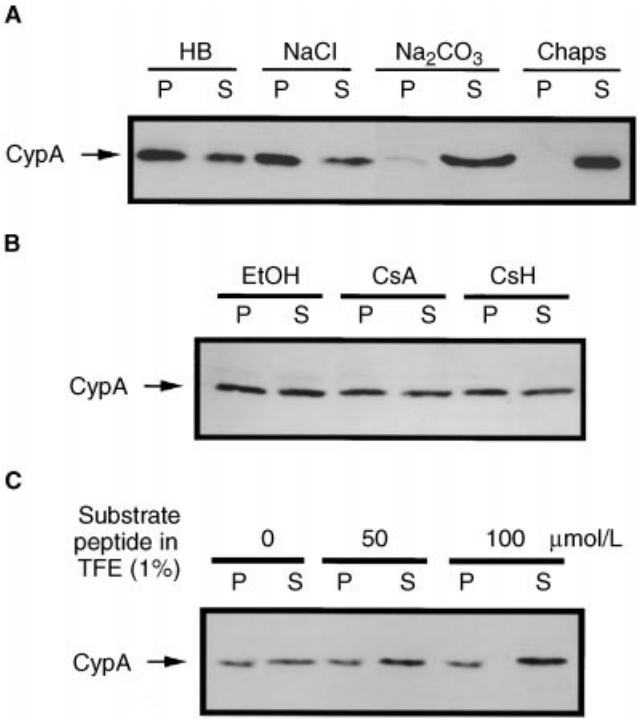

Fig. 4. Extraction of CypA from renal BBMs. (*A*) BBM proteins (45 μg) were incubated in the homogenate buffer (HB) containing 50 mmol/L mannitol and 5 mmol/L HEPES/Tris, pH 7.5, 1 mol/L NaCl, 0.5% CHAPS, or 0.2 mol/L Na_2CO_3, pH 11. (*B*) BBM proteins (45 μg) were incubated in the presence of 2% ethanol as the control (EtOH), 10 μmol/L CsA or CsH. (*C*) Equal amounts of BBM proteins (45 μg) were also incubated in homogenate buffer containing 1% trifluoroethanol, 5 mmol/L LiCl, and 0, 50, or 100 μmol/L of Suc-Ala-Ala-Pro-Phen-pNA peptide. After one hour of incubation at 37°C, the samples were centrifuged. Proteins from the pellet (P) and the supernatant (S) of each sample were separated by SDS-PAGE, and their CypA content was evaluated by Western blot. One representative experiment of three different experiments performed in duplicate is shown.

To determine whether the redistribution of CypA between BBM and soluble fraction from renal cortex was caused by a direct interaction of the drug with CypA, renal BBMs were incubated in vitro with CsA (Fig. 4B). As a control, BBMs were also exposed to cyclosporine H (CsH), a natural cyclosporine that does not interact with CypA. Following the incubations with 10 μmol/L CsA or CsH, BBMs were centrifuged, and the CypA content was evaluated in the pellet or supernatant. Neither CsA or CsH affected the release of CypA from BBM because the CypA content in supernatant and pellet remained similar to the control. These results suggest that chronic CsA administration is required to change the CypA content in BBM.

The Suc-Ala-Ala-Pro-Phen-pNa peptide, a PPIase substrate, was used in an attempt to displace the CypA associated with BBM (Fig. 4C). In contrast with the previous results obtained with CsA, incubation of BBM for one hour with 50 or 100 μmol/L of this CypA substrate increased the release of CypA by 25 and 85%, respectively. These results suggest that under normal conditions, CypA may be released from the membrane by its substrates. In addition, various amounts of CypA were released from BBMs in controls that we performed in Figure 4. This may be explained by the fact that controls in Figure 4 B and C were performed using homogenate buffer containing either 2% ethanol and 1% trifluoroethanol. Thus, the presence of both solvents may interfere with membrane lipids and modify the release of CypA from BBM. This may also indicate the presence of two CypA populations in renal BBM, one that is strongly associated to the membrane, whereas the second population is more affected by both solvents. Further studies are now being carried out to determine and characterize what type of interactions may be involved in the association of CypA with renal BBM.

DISCUSSION

In this study, the subcellular localization of CypA was performed by Western blotting analysis using a pAb directed against CypA. This protein was detected under reducing conditions in all examined subcellular compartments of rat kidney cortex, including the soluble fraction, BBM, mitochondria, microsomes, and the nucleus. Previous studies have shown that CypA was mainly present in the cytosol [19, 20, 31]. However, CypA was also detected in many other subcellular compartments such as the nucleus, mitochondria, microsomes, and Golgi apparatus of various tissues such as the kidney, the liver, and the intestines [29, 30]. Our results show that the CypA concentration is high in renal BBM compared with other IMs and compartments. The absence of enrichment of CypA in soluble fraction or renal BBM is explained by the presence of CypA in various cellular compartments. In normal mammalian tissues, the CypA concentration may account for as much as 0.1 to 0.4% of total protein in the cell [19, 20, 32]. We evaluated the concentration of CypA by immunodetection to be between 2 and 2.5 μg/mg in soluble fraction and in BBM of renal cortex. The presence of CypA in the BBM is not related to an entrapment of CypA during their isolation since RhoGDI, a cytosolic regulatory protein for Rho found in the kidney [33] and of similar size to that of CypA, was detected in soluble fraction, as expected, but not in renal BBMs.

Our results indicate that CypA in both renal fractions was detected as a 18 kD protein when electrophoresis was performed in the presence of the reducing agent β-mercaptoethanol. In the renal soluble fraction, other bands of higher molecular sizes were also revealed by Western blot. These bands were completely absent in renal BBM. These bands could represent other CypA isoforms or CypA oligomeric forms that are resistant to the denaturating conditions used for the gel electrophoresis. When β-mercaptoethanol was omitted during gel

electrophoresis, a strong band that may be related to CypA was revealed only in renal soluble fraction as a >200 kD protein. These results suggest that electrophoresis should be carried out in the presence of a reducing agent for the detection of CypA at the right molecular size by Western blot. In addition, these results indicate that the antigen of CypA in renal BBM is masked in absence of β-mercaptoethanol and that studies that are performed in the absence of reducing agent may not detect CypA in these membranes.

The presence of CypA associated with membranes was previously reported in rat tissues, where it was extractable with urea or CHAPS and represented 5 to 12% of the soluble CypA [34]. In addition, the recruitment of CypA from the mitochondrial matrix into the internal mitochondrial membrane under oxidative stress was also reported [35]. Our results suggest that a portion of CypA in renal cortex is associated with BBMs. To characterize the association and the nature of the attachment of CypA with BBMs, the extraction of CypA from BBMs was performed with various agents. The addition of high ionic strength to the incubation medium did not increase the release of CypA from BBMs, but treatment of BBMs with Na_2CO_3, pH 11, caused a 92% release of CypA associated with these membranes, suggesting that CypA in BBMs is a peripheral membrane protein, since this alkaline treatment does not cause the release of integral membrane proteins. Finally, the detergent CHAPS caused a complete release of CypA associated with BBM, indicating that the attachment of CypA with BBM may involve hydrophobic interactions with either membrane lipids or proteins.

Cyclophilin A has been reported to be highly expressed in the kidney, and it was suggested that CypA may be the main receptor for CsA in renal tissue extracts [22]. Our results indicate that CsA changes the CypA contents of BBM and soluble fraction from renal cortex. These changes are rather specific, as other protein levels such as RhoGDI, Ras, and RhoB in both BBM and soluble fraction remained similar after CsA treatment. This increase in the cytosolic content of CypA is in agreement with a previous report that CsA treatment modifies the cytosolic CypA level, as measured by confocal microscopy and quantitative immunofluorescence [36]. However, in the present study, total CypA content in kidney homogenate from CsA-treated rats remained unchanged compared with control rats, suggesting a redistribution of CypA during CsA administration rather than an increase in total CypA.

In the present study, the photolabeling of CypA with diazirine-CsA was also performed as we previously described for P-glycoprotein [37]. After the administration of CsA to rats, the concentration needed to displace CypA photolabeling by diazirine-CsA was increased in soluble fractions isolated from treated animals, suggesting that the affinity of CypA for CsA had changed, although it remained similar in the BBM. However, when the data were corrected for CsA remaining in the soluble fraction following CsA treatment to the animals, the affinity for CsA was very similar in both populations. On the other hand, the affinity of CypA for CsA measured in BBM from control and CsA-treated rats was similar, indicating that the remaining CsA concentration in membranes was negligible, probably because of the washing steps performed during the preparation of BBM.

In addition to its high affinity for CsA, the cyclophilins possess a PPIase activity [12]. The substrate used in vitro to measure this PPIase activity is the Suc-Ala-Ala-*cis*-Pro-Phen-pNa peptide. In this article, the effect of this substrate and CsA on the release of CypA from BBMs was evaluated. Only the Suc-Ala-Ala-Pro-Phen-pNA peptide increased the release of CypA from renal BBMs. The exact molecular mechanism involved in the release of CypA from BBMs by this peptide remains to be established. However, this result suggests that the binding of this CypA substrate may induce a conformational change in CypA, which reduces its membrane attachment. The molecular mechanism by which CsA treatment alters CypA content remained unclear. However, because the Suc-Ala-Ala-Pro-Phen-pNa peptide substrate caused also a release of CypA from the renal BBM, one possibility that may explain the redistribution of CypA during CsA treatment could be an accumulation of cis-proline residues in the soluble fraction caused by the inhibition of CypA by CsA. This redistribution of CypA could also be related to secondary effects associated with the administration of CsA such as changes in renal tubules. However, the levels of Ras and β-actin as well as the activity of aminopeptidase and various sodium-dependent transporters measured in a previous study using identical CsA treatment [38] were unaffected, suggesting that the effect observed for CypA is not caused by a general degradation of renal BBMs.

In conclusion, our results show that under normal physiological conditions, CypA is also associated with renal BBM. Changes in CypA levels in BBM after CsA administration or after an incubation with one of its substrates indicate also that CypA is in equilibrium between these two subcellular compartments. The role of the CypA associated with the BBM remains to be established. However, it has been shown that CypB may act as a chaperone molecule for membrane proteins [6, 39]. From these latter studies and the common biochemical properties of CypA and B, we could speculate that the CypA associated with BBM may have a similar function for the plasma membrane proteins of the renal proximal tubules. Future studies are needed to identify and establish the molecular mechanism involved in the association of CypA with renal BBMs.

ACKNOWLEDGMENTS

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and from Novartis Canada Inc. to R. Béliveau. A. Laplante received a scholarship from the Fonds pour la Formation des Chercheurs et l'Aide à la Recherche and from the Fonds de Recherche en Santé du Québec. A. Sepehr-Araé received a scholarship from the Natural Sciences and Engineering Research Council of Canada.

Reprint requests to Dr. Richard Béliveau, Laboratoire de Médecine Moléculaire, Université du Québec à Montrèal-Hôpital Ste.-Justine, C.P. 8888, Succursale Centre-ville, Montréal, Québec, Canada H3C 3P8. E-mail: oncomol@er.uqam.ca

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