The Human TRPV6 Channel Protein Is Associated with Cyclophilin B in Human Placenta*

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Transcellular calcium transport in the kidney, pancreas, small intestine, and placenta is partly mediated by transient receptor potential (TRP) channels. The highly selective TRPV6 calcium channel protein is most likely important for the calcium transfer in different specialized epithelial cells. In the human placenta the protein is expressed in trophoblast tissue, where it is implicated in the transepithelial calcium transfer from mother to the fetus. We enriched the TRPV6 channel protein endogenously expressed in placenta together with annexin A2 and cyclophilin B (CypB), which is a member of the huge immunophilin family. In the human placenta TRPV6 and CypB are mainly located intracellularly in the syncytiotrophoblast layer, but a small amount of the mature glycosylated TRPV6 channel protein and CypB is also expressed in microvilli apical membranes, the fetomaternal barrier. To understand the role of CypB on the TRPV6 channel function, we evaluated the effect of CypB co-expression on TRPV6-mediated calcium uptake into Xenopus laevis oocytes expressing TRPV6. A significant increase of TRPV6-mediated calcium uptake was observed after CypB/TRPV6 co-expression. This stimulatory effect of CypB was reversed by the immunosuppressive drug cyclosporin A, which inhibits the enzymatic activity of CypB. Cyclosporin A had no significant effect on TRPV6 and CypB protein expression levels in the oocytes. In summary, our results establish CypB as a new TRPV6 accessory protein with potential involvement in TRPV6 channel function.
is thereby involved in the regulation of the Ca\(^{2+}\) release from intracellular stores (20–23). Concerning the mammalian TRP channels, it was demonstrated by Sinkins et al. (24) that the immunophilin FKBP12 associates specifically with TRPC3, TRPC6, and TRPC7, whereas TRPC1, TRPC4, and TRPC5 interact with FKBP52. The latter immunophilin (FKBP52) was also co-immunoprecipitated from TRPV5-overexpressing cells and showed inhibitory effects on TRPV5 channel activity (25). Like FKBP s cyclophilins (Cyp) are a subfamily of the mammalian immunophilins. Cyclophilins (Cyp) got their name because of their ability to bind to the immunosuppressant cyclosporin A (CsA) (26–28). Immunophilins are a heterogeneous protein family, and the FKBP and Cyp subgroups show no apparent amino acid sequence homology except that they exhibit some sequence specificity for X-proline residues in target-binding proteins. Immunophilins are involved in many intracellular processes, and as peptidyl-prolyl cis/trans isomerases, they accelerate the folding of some proline-containing proteins in vivo and in vitro by catalyzing steps in their initial folding and structural rearrangement. Nuclear functions or co-regulation of cellular receptors and ion channels have been reported and reviewed by Galat (28). Cyclophilins are distributed in different compartments of the cell. Although cyclophilin A resides within the cytosol, cyclophilin B and Cm and the Drosophila homologue NINAA (neither inactivation nor after potential A) comprise cleavable endoplasmic reticulum (ER) signal sequences within their primary structures and accumulate in the ER by an ER retention signal (29, 30), whereas cyclophilin D is mainly found in mitochondria. CypB is not restricted to the ER; it is also localized within the nucleus, where it induces transcription (31), and it is located in the plasma membrane, where it might act as chaperone for correct insertion of plasma membrane proteins (32). Interestingly, the role of CypB is not only limited to intracellular functions; the protein is also secreted into human biological fluids such as blood and milk and serves as messenger for intercellular communication. Allain et al. (33) demonstrated that CypB induces chemotaxis and adhesion to the extracellular matrix of memory CD4\(^+\) T cells (33, 34). Whether CypB co-assembles with ion channels and modifies channel function, however, has not been demonstrated so far.

Our intention was to identify TRPV6 protein expression in human placenta and to identify proteins that might be associated with endogenous TRPV6 and thereby participate in Ca\(^{2+}\) transfer from the maternal to fetal circulation. Most TRP-interacting proteins have been identified so far by yeast two-hybrid screens. We pursued a different approach, the affinity purification of TRPV6 and, in addition, of TRPV6-interacting proteins out of native tissue. We made use of TRPV6 being a Ca\(^{2+}\)-calmodulin-binding protein, and we employed antibodies for TRPV6 to identify TRPV6 proteins in primary human placenta tissue and to enrich these proteins from this tissue. We show that the TRPV6 protein is expressed in human placenta, and we demonstrate for the first time that the immunophilin CypB co-purifies together with the TRPV6 proteins from human placenta microsomal membranes. Both proteins are co-localized in the syncytiotrophoblast layer of the placenta, and the TRPV6-evoked calcium influx in Xenopus laevis oocytes is markedly increased with CypB co-expression.

**EXPERIMENTAL PROCEDURES**

cDNA Constructs/Plasmids—The human TRPV6b/pcDNA3 construct was described by Wissenbach et al. (8). The mouse TRPV5 (NM_001007572) was obtained by reverse transcription-PCR and subcloned in pcDNA3. The human CypB-myc/pcDNA3 plasmid (35) was a generous gift from Toshifumi Matsuyama, Nagasaki University.

Antibodies and Affinity Column Preparation—Polyclonal (429) and monoclonal (20C6, 26B3, 24C3) antibodies against human TRPV6 (36) were used for immunohistochemistry, Western blot analysis, and affinity purification. For affinity column preparation, antibodies were enriched from hybridoma cell culture supernatants with protein G-Sepharose (GE Healthcare). 5–10 mg of affinity-purified monoclonal antibodies were covalently coupled to N-hydroxysuccinimide-Sepharose (GE Healthcare) according to the user manual. The following antibodies/Sepharose were used: polyclonal rabbit anti-CypB antibody (Affinity BioReagents (ABR, PA1-027), polyclonal goat anti-CypB antibody (Santa Cruz Biotechnology (C-15)), horseradish peroxidase-coupled anti-mouse antibody (Dianova), horseradish peroxidase-coupled anti-rabbit antibody (GE Healthcare), calmodulin-Sepharose (GE Healthcare), and horseradish peroxidase-coupled anti-goat antibody (Santa Cruz Biotechnology). Antibodies for TRPV5 were from Alomone Labs and Santa Cruz Biotechnology.

Preparation of Placental Microsomal Membranes and Affinity Chromatography—Placenta obtained from normal term pregnancies were collected immediately after caesarean section or normal delivery from the local hospital and transported on ice to the laboratory. Each sample was performed at 4 °C. Microsomal membrane fractions from human placenta were made by homogenization of the fresh or alternatively frozen (−80 °C) tissue in lysis buffer (100 mM Tris/HCl, pH 8.0, 1 mM MgCl\(_2\)) containing protease inhibitors (10 μM leupeptin, 1 μM pepstatin, 100 μM phenylmethylsulfonyl fluoride) with an Ultraturrax and glass-Teflon potter. After dilution with sucrose buffer (0.25 M sucrose, 10 mM Tris/HCl, pH 7.4) the homogenate was centrifuged at 10,000 × g (RC-5B, Sorvall) for 15 min. The pellet was rehomogenized again with a glass-Teflon potter, and the combined supernatants were centrifuged at 100,000 × g (1.8 Ultracentrifuge, Beckmann) for 45 min. Microsomes were resuspended in 20 mM Tris/HCl-buffer (pH 7.4) and stored at −80 °C.

For membrane protein solubilization, 500 mg of microsomal proteins were homogenized by 20 strokes with a glass-Teflon homogenizer in a final volume of 200 ml of solubilization buffer (0.5% (w/v) sodium deoxycholate, 20 mM Heps, pH 7.4) containing protease inhibitors (10 μM leupeptin, 1 μM pepstatin, 100 μM phenylmethylsulfonyl fluoride). After 1 h of shaking, the solution was centrifuged at 100,000 × g for 45 min. The supernatant was complemented with Triton buffer and Ca\(^{2+}\) (20 mM Heps, pH 7.4, 100 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM CaCl\(_2\)) to a final volume of 1500 ml and immediately transferred over a 20-mL calmodulin-Sepharose column. The solution circulated overnight with a flow rate of 3–4 ml/min. After washing with Triton/Ca\(^{2+}\) buffer, the calmodulin column was eluted with Triton buffer supplemented with EGTA (20 mM...
Cyclophilin B Enhances TRPV6-mediated Calcium Transport

Hepes, pH 7.4, 100 mM NaCl, 0.1% (v/v) Triton X-100, 2 mM EGTA). This eluate was transferred to a monoclonal TRPV6-antibody (20C6)-column preequilibrated with Triton/EGTA buffer and circulated for 4 h (flow rate 1 ml/min). For negative control purification, the calmodulin column eluate was transferred over a column with covalently bound mouse IgG to N-hydroxysuccinimide (NHS)-coupled Sepharose or ethanolamine-blocked NHS-Sepharose. Proteins were eluted from antibody and negative control columns with the antigenic peptide (1.3 mg/ml) in Triton X-100 buffer plus EGTA. The eluate was concentrated by ultrafiltration with Vivaspin molecular weight cutoff 10,000 (Vivascience) and stored at −20 °C.

The isolated proteins were denatured with loading buffer at 37 °C for 30 min, separated in 4–12% Bis-Tris gel with a MOPS buffer system (NUPAGE®-Novex, Invitrogen) or 4–12% Tris-glycine SDS-PAGE, and visualized with colloidal Coomassie (34% (v/v) methanol, 2% (v/v) phosphoric acid, 17% (w/v) ammonium sulfate, 0.066% (w/v) Coomassie G250) or silver-stained (37). Protein bands were cut and prepared for nano-liquid chromatography-MS and MALDI mass spectrometry.

**MALDI and ESI Mass Spectrometry and Data Base Research—**

The excised bands were alternately washed twice with 50 mM ammonium hydrogen carbonate (solution A) and 50% (v/v) ammonium hydrogen carbonate (50 mM), 50% (v/v) acetonitrile (solution B). Reducing of disulfide bridges was performed by incubation for 30 min at 56 °C in 50 mM ammonium hydrogen carbonate, 10 mM diithiothreitol followed by carboxymethylation for 30 min at room temperature in darkness in 50 mM ammonium hydrogen carbonate with 5 mM iodoacetamide. Gel pieces were washed twice alternating with solution A and B and then dried. The in-gel digestion was proceeded by incubating the pieces with 4.5 μl of a 25 μg/ml solution of porcine trypsin (Promega) at 37 °C overnight. The resulting peptides were extracted by shaking the gel in 0.1% trifluoroacetic acid. Extracted peptides were concentrated with PerfectPure C-18 Beads with captured immunocomplexes were pelleted, washed 3 times with 1 ml of solubilization buffer, and eluted with 30 μl of 2× SDS loading buffer for 20 min at 61 °C, and the proteins were separated by SDS-PAGE. 75 μg of control protein lysates or 25 μl of immunoprecipitated proteins were loaded per lane and after electrophoresis transferred to a nitrocellulose membrane (Hybond C, GE Healthcare) by tank-blotting in Tris-glycine buffer (1 h, 350 mA). The membrane was stained with 0.5% (w/v) Ponceau red in 1% acetic acid and blocked with 5% (w/v) nonfat dried milk in TBS (150 mM NaCl, 50 mM Tris, pH 7.5) for 1 h. The immunoblots were incubated with primary antibodies overnight at 4 °C, washed 3 times in TBS, incubated with the corresponding secondary antibody coupled to horseradish peroxidase for 1 h at room temperature, washed 3 times, and finally, developed by chemiluminescence with the Western Lightning Chemiluminescence Reagent plus (PerkinElmer Life Sciences).

**Enrichment of Apical and Basolateral Synctiotrophoblast Membranes of Human Placenta, and Glycosidase Treatment of the Apical Membrane Fraction—**Placenta obtained from caesarean section at a local women’s hospital was carried to the laboratory on ice. Maternal decidua was removed from the placenta, and the villous tissue was scraped from blood vessels and other connective tissue and grown with an Ultraturrax in 3
Cyclophilin B Enhances TRPV6-mediated Calcium Transport

JUNE 27, 2008 • VOLUME 283 • NUMBER 26

JOURNAL OF BIOLOGICAL CHEMISTRY

volumes of lysis buffer (10 mM Tris-HCl, 250 mM sucrose, 1 μM pepstatin, 10 μM leupeptin, 100 μM phenylmethylsulfonyl fluoride, pH 7.4) 5 times with 2-s intervals. The following procedure was adapted from Jimenez et al. (39). Membrane fractions were characterized by enzyme assays and Western blot analysis with marker proteins to evaluate the enrichment of apical plasma membranes and to identify contamination with ER and mitochondrial membranes. Therefore, the activity of alkaline phosphatase, an epithelial apical membrane marker, was measured according to Tietz (40). The activity of the inner mitochondrial membrane marker succinate dehydrogenase was assayed as described by Munujo et al. (41). Western blot experiments were performed with antibodies against the basolateral membrane marker protein adenylate cyclase (R-32, Santa Cruz Biotechnology) and the human ER marker protein calnexin (H-70, Santa Cruz Biotechnology). For glycosidase digestion, 400 μg of protein from the apical membrane sample LA was treated with peptide N-glycosidase F or endoglycosidase H glycosidase (New England Biolabs). The digestion was carried out according to the protocol provided from the manufacturer. The sample was stored in 2× SDS loading buffer at −20 °C before SDS-gel electrophoresis.

Immunohistochemistry—The method was accomplished as described by Bernucci et al. (11). Human placenta was obtained immediately after caesarean section and carried on ice to the laboratory. Small pieces (about 1 cm³) were cut, and the blood was washed away with ice-cold 0.9% (w/v) NaCl solution. The tissue was quickly frozen in Cryogel with liquid nitrogen. 10-μm cryosections were cut and collected on superfrost slides. After air drying, the sections were fixed in 4% paraformaldehyde for 30 min at room temperature. The slides were then rinsed 3 times in TBS buffer for 10 min, and the sections were blocked overnight in TCT buffer (0.7% carrageenan, 0.5% Triton X-100 buffered in TBS). Carrageenan is a sulfated linear polysaccharide that is usually used as a blocker in immunohistochemistry (42). After blocking, excess TCT buffer was removed with a soft tissue. All primary antibodies for the staining were diluted with deionized water at the desired concentrations: anti-TRPV6 antibodies (429, 80 μg/ml; 24C3, 160 μg/ml; 26B3, 50 μg/ml), anti-human placental alkaline phosphatase polyclonal antibody (Sero Tech AHP 537Ht, ready to use), anti-cytokeratin 7 monoclonal antibody (Bioprime CK507, ready to use), and anti-CypB polyclonal antibody (ABR PA1–027, 1:100). The primary staining was carried out at room temperature for 2 h, and the slides were rinsed in TBS 3 times each for 10 min. The sections were then stained for 1 h at room temperature with goat anti-mouse or anti-rabbit secondary antibodies conjugated with Cy3 or Alexa488 diluted 1:200 in deionized water. After rinsing in TBS, coverslips were embedded, and the slides were mounted on a confocal microscope (LSM 510, Zeiss, Jena) and viewed with LSM Image Browser (Zeiss).

Oocytes Preparation, cRNA Synthesis, and Injection—X. laevis females were purchased from Horst Kähler, Hamburg, Germany. Oocytes were isolated and singularized by collagenase (collagenase A, Roche Applied Science) treatment in Ca²⁺-free oocyte Ringer solution at room temperature for 2 h. Oocytes were kept in oocyte Ringer solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 5 mM HEPES, pH 7.4) overnight before injection. The cRNA preparation and injection was described earlier (Becker et al. (43)). In brief, the pcDNA3 vectors containing TRPV6 cDNA (8), TRPV5 cDNA, and CypB cDNA (35) were linearized downstream of the target gene with NotI (in the case of TRPV6), Nhel (in the case of TRPV5), and XhoI (in the case of CypB), respectively. In vitro transcription was performed with the mMESSAGE mACHINE T7 Kit (Ambion, Austin, TX). Defolliculated X. laevis oocytes were injected with either 14 nl of water or cRNA solution. 3.5 ng of TRPV6 cRNA alone or together with 3.5 or 7 ng of CypB cRNA were injected per oocyte. Oocytes were incubated in Barth’s solution (88 mM NaCl, 2.4 mM NaHCO₃, 1 mM KCl, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 5 mM Tris-HCl, pH 7.4). The calcium assays were carried out 3 or 4 days after cRNA injection. 

45Ca Uptake Assay—[45Ca²⁺] uptake assay in oocytes was adapted as described by Peng et al. (44) and Jiang et al. (45) at days 3 and 4 after injection. Before the uptake assay experiment, oocytes were observed under a binocular microscope. The oocytes were rinsed with uptake buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂) and then immediately incubated for 2 h at room temperature in uptake buffer containing 10 μCi/ml 45CaCl₂. After gentle washing the oocytes 6 times with ice-cold uptake solution without 45CaCl₂, the oocytes were dissolved in 10% SDS solution, and the incorporated radioactivity was determined by scintillation counting. The calcium uptake data are presented as the mean values ± S.E. Oocyte calcium uptake data were obtained from at least three individual animals with 7–15 oocytes/group.

Immunohistochemistry of the X. laevis Oocytes—3 days after cRNA injection, the oocytes were fixed in 4% paraformaldehyde for 30 min and washed with PBS 3 times for 10 min. Subsequently, the oocytes were treated with 100% methanol at −20 °C for 20 min to permeabilize the cells. After 3 washings with PBS, oocytes were incubated in blocking buffer (3% bovine serum albumin, 1% goat serum, and 0.1% Triton X-100) for 1 h at room temperature. The oocytes were then stained with primary antibodies overnight at 4 °C and diluted in PBS containing 1% bovine serum albumin and 0.1% Triton X-100. After washing with PBS, the cells were subsequently incubated with secondary antibodies coupled to Alexa488 or Cy3 diluted 1:200 in PBS and incubated for 1 h at room temperature. The cells were again washed 3 times with PBS and observed with a confocal laser scanning microscope (LSM 510, Zeiss, Jena) using a 40× water immersion lens.

RESULTS

Affinity Purification of the Human TRPV6 Channel Protein and Associated Proteins—The human TRPV6 protein consists of 725 amino acids with a calculated molecular mass of 83 kDa. TRPV6 was purified by two consecutive affinity chromatography steps, calcium/calmodulin and by immunoaffinity chromatography. As we have shown previously (46), the C terminus of TRPV6 binds calmodulin in a Ca²⁺-dependent manner; we used calcium/calmodulin chromatography to enrich TRPV6 (Fig. 1, upper blot) from solubilized human placenta microsomal proteins. The amount of endogenous TRPV6 is relatively low because we were not able to detect the protein when apply-
Antibody (Fig. 2) was not found in fractions of the negative control (Fig. 1, lower blot). CypB has not been described as a calmodulin-binding protein so far, and although most of the CypB protein eluted in the first two fractions, it was also found in fractions 3 and 4, where the highest amount of the TRPV6 protein was detectable. Using the monoclonal antibody column as a second step, CypB was always co-purified with TRPV6 and identified by Western blot analysis and/or mass spectrometry analysis in eight independent purifications (Fig. 3A, left side). CypB was not detectable in the purifications that were undertaken without the specific TRPV6 antibody (Fig. 3A, right). CypB comprises 216 amino acids, where the N-terminal amino acids 1–25 resemble an ER signal sequence and the C terminus of the protein contains an ER retention sequence. The mass spectrometric analysis shows sequence coverage of 51% (Fig. 3B). As described previously (30), CypB is retained in the ER by the C-terminal peptide 

\[ \text{AcKVEKPKFAIK215} \] 

by peptide mass fingerprint analysis. Only the very last glutamate residue predicted from the published primary structure could not be detected by MALDI-TOF measurements, most probably because of the use of trypsin as protease (Fig. 3). In addition to CypB, the annexin 2 isoform 1 (gi:50845388) was also co-purified with TRPV6 microsomal membranes. Annexin 2 was detected in 4 of 8 purifications (Fig. 2, B and C, white arrows), and we could detect the protein by MALDI-MS analysis and/or Western blot analysis (data not shown). As has been reported by Kristoffersen et al. (48), annexin 2 and its subunit p11 (S100A10) are expressed on the surface of microvillus syncytiotrophoblast plasma membranes. Annexin 2/p11 apparently interacts with TRPV5 and TRPV6 via the p11 protein, which predominantly forms a heterotetrameric complex together with annexin 2, when heterologously expressed in oocytes (49).

Next we demonstrated the interaction of CypB and TRPV6 by an independent method, by co-immunoprecipitation using human placenta microsomal proteins and a polyclonal TRPV6 antibody (Ab429). In the precipitate CypB (Fig. 4A) and TRPV6 was retained, but neither TRPV6 nor CypB was found in negative controls. We also used CypB antibodies which are suitable for detection of the CypB protein after affinity purification in Western blots (Figs. 1 and 3) for co-immunoprecipitation, but they then did not precipitate CypB or TRPV6 (data not shown).

CypB is Co-expressed with TRPV6 in Different Human Tissues—So far our data obtained by two independent methods indicate that CypB and TRPV6 specifically interact. To test co-expression of CypB and TRPV6, we incubated a multi-tissue Western blot with TRPV6 and CypB antibodies (Fig. 4B). The CypB protein is readily detectable in human liver (Fig. 4B, lane 4), placenta (lane 16), spleen (lane 7), stomach (lane 9), small intestine (lane 10), and colon (lane 11). Only weak expression was found in lung (lane 5), pancreas (lane 6), uterus (lane 13), prostate (lane 14), and testis (lane 15). All tissues expressed
FIGURE 2. Purification of TRPV6 and associated proteins from human placenta microsomal membranes. A, Western blot analysis with TRPV6-specific antibody 429 after a two-step purification procedure (+): Ca\(^{2+}\)-calmodulin and immunoaffinity chromatography (see "Experimental Procedures") —, immunoaffinity chromatography was performed with nonspecific IgGs, covalently linked to Sepharose. HEK TRPV6, lysate from TRPV6-expressing human embryonic kidney cells.

B, colloidal Coomassie-stained SDS-PAGE with proteins enriched by the two-step procedure (+) or by using the nonspecific IgG column as a second step (—). Protein bands of both lanes (+) and (—) were cut and transferred to mass spectrometric analysis. Black arrow, TRPV6; gray arrow, CypB; white arrow, annexin 2 (ANX2). These proteins were not found in the negative control (—).

C, colloidal Coomassie-stained SDS-PAGE of proteins obtained by the two-step procedure separated by SDS-PAGE in the absence of reducing agents (right panel) and a corresponding Western blot (left panel). TRPV6 runs as high molecular mass complex (>170 kDa) in the absence of β-mercaptoethanol in the Western blot and was identified after its isolation out of the gel by MALDI-TOF analysis (right, black arrow). Only small amounts of the glycosylated and non-glycosylated (100–120 kDa) TRPV6 protein (left, black arrows) were detectable under these conditions. Annexin 2 (white arrow) and CypB (gray arrow) were detected by MALDI-TOF analysis. D, coverage of the amino acid sequence of TRPV6 by the peptides identified by peptide mass fingerprint analysis after MALDI-TOF analysis (red). The C-terminal peptide identified by MS-MS analysis is shown in green. E, the MALDI-MS/MS spectra of a peptide unique for TRPV6 from the processed TRPV6 protein band shown in B. The amino acid sequence derived from the mass differences of the b-ion series is given in N- to C-terminal direction.
Cyclophilin B Enhances TRPV6-mediated Calcium Transport

**FIGURE 3.** A, CypB is co-purified with the human TRPV6 protein after Ca\(^{2+}\)-calmodulin and immunoaffinity chromatography. Western blot analysis with the CypB antibody of two independent TRPV6 affinity purifications (left, +) and the corresponding negative controls are shown (right, −). B, coverage of the amino acid sequence of CypB (band from gel shown in Fig. 2B) by the peptides identified by peptide mass fingerprint analysis after MALDI-MS analysis (red). Green-colored amino acid residues were identified through MALDI and ESI-MS analysis, and blue-colored residues were detected through ESI-MS. The signal peptide of CypB (gi:4758950) is underlined (N terminus), and the ER-retention signal is boxed.

glyceraldehyde-3-phosphate dehydrogenase, which was used as a loading control (data not shown). After incubating the multi-tissue blot with the poly- and monoclonal TRPV6 antibodies, the TRPV6 protein was not detectable (data not shown), not even in the placenta (lane 16). The protein fractions applied were obtained from tissue homogenates and most probably did not contain sufficient amounts of TRPV6. However, comparing the TRPV6 transcript expression described formerly (36, 50–52) with the CypB expression shown in Fig. 4B suggests co-expression of both gene products in spleen, stomach, intestine, pancreas, and prostate in addition to placenta.

**TRPV6 and CypB Co-localize Intracellularly and Near Apical Membranes of Human Placenta Syncytiotrophoblasts**—To locate the TRPV6 protein in placenta, apical and basolateral membrane fractions of syncytiotrophoblasts were enriched by ultracentrifugation and sucrose gradient centrifugation steps (Fig. 5A). The success of the enrichment was analyzed by assaying marker enzymes and by Western blots of marker proteins (Fig. 5, B–D). The results obtained were in agreement with those described previously (39); they showed that alkaline phosphatase activity is enriched about 20-fold in fractions containing apical membranes (LA, A, and mA), whereas only low activity of alkaline phosphatase was found in fractions primarily containing basolateral membranes (Bp, mB, B, and B1). Adenylate cyclase (Fig. 5C) was only present in basolateral membrane fractions (mB, B) and the homogenate (H). The apical membrane fraction LA was virtually free from ER membranes (Fig. 5B), whereas the basolateral membrane mB, B, Bp, and Mp contained ER (Fig. 5B) and mitochondrial membranes (Fig. 5D, white bars). ER content was highest in the basolateral membrane fractions mB, B, and Bp when probed with the antibodies for the ER membrane marker calnexin (Fig. 5B). Western blot analysis showed that TRPV6 is expressed as a mature glycosylated (g) and, in addition, non-glycosylated (n,g) protein (Fig. 5E). Whereas the mature glycosylated TRPV6 is primarily detectable in purified apical membranes (LA, A) and only to a minor extent in basolateral membrane fractions (B), the non-glycosylated form is predominantly present in fraction B, which in contrast to fraction LA contains a considerable amount of ER membranes. At the same time, non-glycosylated TRPV6 is also present in other fractions enriched with ER membranes, such as Bp and mB. We also tested whether the glycosylated TRPV6 protein can be deglycosylated by using fraction LA, which contains the highest amount of glycosylated TRPV6. The glycosylated TRPV6 protein could be deglycosylated by peptide N-glycosidase F but not by endoglycosidase H (Endo H; Fig. 5G). In summary, these data indicate that the non-glycosylated TRPV6 is mainly located in the ER, whereas the mature glycosylated form is primarily located in the apical membrane of microvilli. At the same time CypB is more abundant in the ER-containing membrane fractions (Fig. 5F) but is also present in the apical membrane fractions (LA), consistent with previous reports (28).

Next we performed immunohistochemistry using placenta sections and monoclonal and polyclonal antibodies for TRPV6 (36), alkaline phosphatase, cytokeratin 7, and CypB. As can be seen in Fig. 6, B, E, and K, the polyclonal and monoclonal TRPV6 antibodies stain proteins in the villous core and also in the syncytiotrophoblast layer. Additionally, fluorescent co-staining experiments with the cytoskeletal protein cytokeratin 7 (Fig. 6A), a syncytiotrophoblast marker, and alkaline phosphatase, the apical microvilli membrane marker, were performed. TRPV6 staining was apparent in the syncytiotrophoblast layer, but the protein is also co-localized in or near the apical microvilli membranes together with placental alkaline phosphatase (Fig. 6, D–F). The optical resolution of the confocal microscope, however, does not allow distinguishing between antibody staining of membrane proteins and proteins localized near the membrane. Fig. 6, H and I, show that CypB antibody staining is not only present in the syncytiotrophoblast layer but is also found in cells within the villous core. The double staining of TRPV6 and CypB (Fig. 6, J–L) indicates that the two proteins co-localize mainly intracellularly in the syncytiotrophoblast layer. In control sections, to test nonspecific binding of the secondary antibody, it was incubated in the absence of primary antibodies, and no significant staining was apparent (data not shown). In summary, the results obtained by membrane fractionation and immunohistochemistry demonstrate that TRPV6 and CypB are co-localized intracellularly, probably in the ER, but also at the apical membrane of the human placenta syncytiotrophoblast, which builds the fetomaternal barrier.
Co-expression of CypB and TRPV6 Activates the TRPV6-mediated Calcium Uptake in X. laevis Oocytes—TRPV6 channels carry Ca\(^{2+}\) (7, 8), and the interaction between CypB and TRPV6 was further substantiated by using a calcium uptake assay in X. laevis oocytes. Oocytes were injected with human TRPV6 cRNA alone or co-injected with human TRPV6 and CypB cRNAs, and the TRPV6 surface expression of the oocyte was detected by antibodies 3 days after injection (Fig. 7A). In addition, the glycosylated and non-glycosylated TRPV6 proteins were detected in Western blots after immunoprecipitation.
Cyclophilin B Enhances TRPV6-mediated Calcium Transport

From oocyte lysates (Fig. 7B, left side), Notably, oocytes endogenously express a cyclophilin as suspected after Western blot analysis with the human CypB antibody (Fig. 7B, right side). We, therefore, also performed MS/MS analysis of oocyte lysates proteins in the range of 15–22 kDa shown in Fig. 8A. This lead to the identification of four peptides in one protein band that are part of Xenopus cyclophilin B (XCypB, gi:148237181, Fig. 8B, boxed sequence). We also observed that the XCypB protein, which has a theoretical Mr of 23.8 kDa, migrates slightly slower in comparison to human CypB (Mr, 22.7 kDa) expressed in placenta microsomes; in addition, both proteins migrate slightly faster than their expected size maybe because of posttranslational modifications (Fig. 8A). The XCypB protein shares 86% identical amino acids with the human CypB (Fig. 8B). Especially the absolute C termini, which contain the epitope of the used anti-human CypB antibody, are identical between the two species.

In TRPV6-expressing oocytes, the Ca$^{2+}$ uptake increased 9.8-fold as compared with water-injected oocytes (Fig. 7C). This TRPV6-induced Ca$^{2+}$ uptake was further increased by 35 ± 5.6% after coinjection of 3.5 ng of CypB or 60 ± 9.8% after coinjection of 7 ng of CypB cRNA, respectively (Fig. 7D). No significant calcium uptake into oocytes was observed when only 3.5 or 7 ng of CypB cRNA were injected, indicating that human CypB itself does not promote endogenous Ca$^{2+}$ uptake processes in the oocyte (data not shown). The immunosuppressive drug CsA is known to bind to CypB and to inhibit its enzyme activity (29). The incubation of oocytes in the presence of 30 μM CsA did not influence TRPV6-induced Ca$^{2+}$ uptake into oocytes injected with TRPV6 alone (Fig. 7E, TRPV6 versus TRPV6 in CsA). However, it significantly reduced the Ca$^{2+}$ uptake in TRPV6/CypB co-expressing oocytes to levels very similar to those obtained in oocytes only expressing TRPV6, indicating that CsA inhibits the stimulatory effect of CypB on TRPV6-mediated calcium uptake.

To investigate whether the activation of CypB induces a higher expression of the TRPV6 protein, we immunoprecipitated the TRPV6 protein under the various experimental conditions (Fig. 7F); however, the protein levels of TRPV6 (Fig. 7F, left side) and CypB did not change significantly after TRPV6/CypB co-expression or CsA treatment (Fig. 7F, right side), respectively. Taken together, these results demonstrate that CypB does not influence TRPV6 protein expression in Xenopus oocytes but specifically increases TRPV6-mediated Ca$^{2+}$ uptake. This increase can be reversed by preincubation of oocytes with CsA.

Because of a considerable amino acid sequence identity of TRPV6 and TRPV5 (75%), we also tested whether CypB may have an effect on TRPV5 channel function. We, therefore, injected oocytes with mouse TRPV5 and observed a high calcium uptake that was comparable with the TRPV6 uptake rates. But after coinjection of human CypB, the TRPV5 evoked calcium uptake was decreased in a concentration-dependent manner (Fig. 7G), and additionally, in contrast to TRPV6, the
FIGURE 7. CycB enhances TRPV6-mediated calcium uptake in X. laevis oocytes and decreases TRPV5 activity. A, immunocytochemical staining of TRPV6-expressing oocytes 3 days after cRNA injection (left) and water-injected oocytes (right). B, Western blot of proteins obtained from oocytes which were injected with water or TRPV6 cRNA. Left panel, proteins immunoprecipitated with the antibody for TRPV6. The glycosylated (upper band) and non-glycosylated (lower band) TRPV6 protein was expressed in oocytes. Right panel, oocyte lysates probed with anti-human CycB antibody. An endogenous XCycB was detected. Calcium uptake of water (mock) or TRPV6 cRNA (–) or TRPV6 plus CycB cRNA (–) injected oocytes was measured 3 or 4 days after injection. TRPV6-evoked calcium uptake was increased by CycB co-expression in a concentration-dependent manner. Data shown were obtained from the TRPV6 + CycB co-injected oocytes at day 4 after injection. The amount of cRNA was 3.5 ng for TRPV6 and 3.5 or 7 ng for CycB, respectively. E, CsA effect on TRPV6 or TRPV6/CycB-expressing oocytes. The TRPV6 and TRPV6/CycB-injected cells were maintained for 4 days in the absence or presence of 30 μM CsA. F, expression levels of TRPV6 and the Myc-tagged human CycB in the oocytes were not significantly affected by TRPV6/CycB co-expression or CsA treatment. Shown is a representative Western blot analysis of the immunoprecipitated TRPV6 protein detected with antibody 26B3 (left side) and a Western blot of cell lysates with an anti-human CycB antibody (right side). On the right side, the upper band corresponds to Myc-tagged human CycB (~24 kDa), and the lower band (~21 kDa) corresponds to the oocyte endogenous protein (XCycB), which was also recognized by the anti-human CycB antibody. G, calcium uptake of water (mock) or TRPV5 cRNA or TRPV5 plus CycB cRNA-injected oocytes was measured 3 or 4 days after injection. TRPV5-evoked calcium uptake was decreased by CycB co-expression in a concentration-dependent manner. Data shown were obtained from the TRPV5 + CycB co-injected oocytes at days 3 and 4 after injection. The amount of cRNA was 3.5 ng for TRPV5 and 3.5 or 7 ng for CycB, respectively. H, CsA effect on TRPV5 or TRPV5/CycB-expressing oocytes. The TRPV5 and TRPV5/CycB-injected cells were maintained for 3 and 4 days in the absence or presence of 30 μM CsA.
Cyclophilin B Enhances TRPV6-mediated Calcium Transport

TRPV5-injected oocytes were sensitive to CsA treatment (Fig. 7H). Therefore, on the one hand CsA treatment leads to a 24 ± 5.3% reduction in calcium uptake, but on the other hand, the inhibition of calcium uptake after TRPV5/CypB coinjection was not further sensitive for CsA treatment (Fig. 7H), suggesting a different mechanisms for the potential TRPV5/CypB interaction.

DISCUSSION

Our data demonstrate that TRPV6 is expressed in human placenta, and just like overexpressed TRPV6 (36, 46), it is capable of Ca^{2+}-calmodulin binding. We enriched the TRPV6 protein from solubilized membrane protein fractions from human placenta using a two-step purification procedure. This purification includes a Ca^{2+}-calmodulin and an immunoaffinity column. TRPV6 proteins co-eluted with CypB and annexin A2. The latter was recently identified as a TRPV5/6-interacting protein by a yeast two-hybrid screen (49). The annexin A2-p11 complex seems to be necessary for proper trafficking of the TRPV5 and TRPV6 channels to the plasma membrane of transfected HEK293 cells. We identified annexin A2 in 50% of the two-step affinity purifications. Whether the endogenously expressed TRPV6 channel protein is also connected to the annexin A2-p11 tetramer complexes or annexin A2 itself in syncytiotrophoblast plasma membranes of human placenta is not known, and our mass spectrometric and Western blot results (data not shown) do not rule out either possibility. In contrast to annexin A2, the CypB protein was always co-eluted with the TRPV6 ion channel. CypB belongs to the family of immunophilins but has not been shown so far to co-purify with ion channel proteins. During calmodulin chromatography we observed that CypB elution does not exactly pursue the elution of TRPV6 (Fig. 1). This finding may indicate that TRPV6 is the limiting component of a TRPV6/CypB complex and that additional calmodulin-binding proteins may exist that are capable of interacting with CypB. In fact, CypB is much more abundant than TRPV6, and only a minor fraction might be sufficient to saturate interaction with TRPV6.

To investigate the expression pattern of both proteins in the placenta, we isolated membrane fractions of syncytiotrophoblast tissues and investigated the expression of CypB and TRPV6 by Western blot analysis and immunostaining. In line with previous reports, CypB is mainly localized in the ER. The native TRPV6 protein is glycosylated like the heterologously expressed protein (36) and is found at the apical membrane of syncytiotrophoblasts, where it could act as an entry gate for Ca^{2+}. When expressed in Xenopus oocytes, TRPV6 acts as a Ca^{2+} entry channel, and the TRPV6-induced Ca^{2+} uptake in oocytes was significantly increased after CypB co-expression. This increase was reversed by incubating oocytes in the presence of CsA. CsA blocks the enzymatic activity of the peptidyl-prolyl cis/trans isomerase activity of CypB, suggesting that this CypB enzyme activity is essential to increase TRPV6-induced Ca^{2+} uptake in oocytes. Alternatively, CsA may bind to a region of CypB that is required for TRPV6 interaction. Although endogenous Xenopus cyclophilin is highly abundant in comparison to the injected Myc-tagged human CypB, it seems to have no stimulatory effect on the TRPV6-evoked Ca^{2+} uptake. In oocytes, which express only TRPV6, incubation with CsA does not change the Ca^{2+} uptake. We suggest that the human TRPV6 does not interact with the Xenopus cyclophilin B protein, which is endogenously expressed in oocytes, although its protein sequence is similar to human cyclophilin B. We attribute that the human TRPV6-cyclophilin B interaction depends on the 14% of amino acids, which are not identical to the Xenopus cyclophilin.

Although we have no biochemical evidence for an interaction of CypB with TRPV5 so far, we tested the closest TRPV6 relative, TRPV5, in the oocyte expression system. TRPV5-evoked calcium uptake is also markedly changed after CypB coexpression, but in contrast to TRPV6, the CypB expression led to a reduction of calcium uptake. This reduced TRPV5 calcium uptake is not sensitive to CsA treatment, although CsA affected the calcium uptake in the absence of coinjected CypB cRNA. Possibly the TRPV5 protein expression is reduced after
Coinjection of CypB-cRNA. Commercially available antibodies for TRPV5 did not recognize the TRPV5 protein in oocyte lysates. The observed 24% reduction in calcium uptake in the presence of CsA in oocytes only injected with the TRPV5-cRNA may indicate that TRPV5 interacts with endogenous frog CypB or other frog cyclophilins that may be sensitive to CsA. The finding that after coinjection of hCypB- and TRPV5-cRNA the calcium uptake was reduced and no longer influenced by CsA treatment argues for a mechanism quite different to the one observed for TRPV6. Maybe this regulation mirrors the situation in cells which express both TRPV5 and TRPV6, such as placenta and maybe kidney and pancreas (33).

CypB was originally described as an ER protein (29, 30), and we also found an almost complete ER retention signal in the coinjected protein by mass spectrometric analysis. It is possible that during the purification procedure different populations of TRPV6/CypB complexes were purified; one TRPV6/CypB population, comprising the ER retention sequence, was retained in the ER, and another population, without the ER retention sequence, is localized in the plasma membrane. Our immunohistochemical data support this hypothesis, which is also in agreement with previous reports showing that CypB is transferred to the plasma membrane, where it is suggested to act as chaperone for plasma membrane proteins (32).

Interestingly, we did not identify other TRP channel proteins by our purification procedure, although it was supposed that TRPV5 is also expressed in human placenta as part of heterooligomeric TRPV5/TRPV6 channels (47). Apparently, the choice of buffers, detergent, and ionic conditions employed here might interfere with TRPV6/TRPV5 interaction, although there is also the possibility that endogenous TRPV6 and TRPV5 do not interact. In summary, our results provide strong evidence that CypB is associated with TRPV6 proteins and regulates TRPV6-induced Ca$^{2+}$ uptake by a CsA-sensitive process.

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Cyclophilin B Enhances TRPV6-mediated Calcium Transport

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