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A Novel, Single, Transmembrane Protein CATSPERG Is Associated with CATSPER1 Channel Protein[1]

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ABSTRACT

All four CATSPER channel pore-forming subunits (CATSPER1–4) are localized in the sperm principal piece. They form an alkalization-activated Ca^{2+}-permeable channel and are required for sperm-hyperactivated motility, egg coat penetration, and male fertility. Unlike many other ion channels, the composition of the CATSPER protein complex is poorly defined. Herein, we describe the novel protein CATSPERG associated with the CATSPER complex. CATSPERG is predicted to be a single transmembrane-spanning protein with a large extracellular domain and a short intracellular tail. Like all the CATSPERs and the previously identified CATSPER-associated protein CATSPERB, CATSPERG is only expressed in testis and is localized in the sperm principal piece. In CATSPER1-deficient sperm, the CATSPERG protein (but not the K^+ channel protein KCNU1) is also lost. Together with previous findings, our data suggest that the CATSPER protein complex contains pore-forming proteins and two additional proteins (CATSPERB and CATSPERG) and that the trafficking and/or assembly of these proteins depends on CATSPER1.

calcium, gamete biology, signal transduction, sperm motility and transport

INTRODUCTION

Sperm cells in animals ranging from sea urchins to humans use Ca^{2+} as a messenger to control cellular processes such as capacitation, motility, and the acrosome reaction [1]. Proteins of many Ca^{2+}-permeable ion channels, including the highly selective voltage-gated Ca^{2+} channels (Ca_Vs), the less selective transient receptor potential channels, and the cyclic nucleotide-gated channels, have been detected in testis and sperm [2–4]. In addition, these proteins are found in other cell types such as neurons and muscle cells. The in vivo significance of these proteins to sperm physiology and fertilization remains unclear, as targeted disruption of these channel genes in mice resulted in no obvious male fertility defect [5–10]. Sperm also have a family of sperm-specific ion channels, CATSPER [11, 12]. CATSPER channels have characteristics of other classes of cation channels. Like voltage-gated K^+ channels (K_Vs), the CATSPER pore-forming subunits are six transmembrane (TM)-spanning proteins, but CATSPER selectivity filter sequences are similar to those of Ca_V channels (24 TM) [13]. Mammals have four *CATSPER* genes (*CATSPER1–4*), each of which is required for sperm-hyperactivated motility and male fertility, as shown by targeted gene disruption in mice [11, 12, 14]. In addition, mutation in the *CATSPER2* gene is implicated in male infertility in humans [15]. CATSPER channels are required for the Ca^{2+} influxes activated by an alkaline depolarizing medium [16] and cyclic nucleotides [13]. Calcium ions entering the channels in the principal piece trigger intracellular Ca^{2+} concentration increases in the midpiece and head through unknown mechanisms [17]. The CATSPER proteins presumably form an alkalization-activated Ca^{2+}-permeable channel in sperm, as disruption of the *Catsper* genes in mice also led to the elimination of such a channel current [11, 14, 18]. Attempts to express functional CATSPER channels in heterologous systems have been unsuccessful.

In general, a complete ion channel complex is composed of pore-forming proteins and one or more auxiliary subunits [19]. For example, Ca_V channels consist of a pore-forming $\alpha 1$ subunit that determines the ion selectivity, a β subunit (an intracellular protein), a single TM-spanning $\alpha 2\delta$ subunit, and a multiple membrane-spanning γ subunit [20]. Similarly, voltage-gated Na^+ (Na_V) channels are composed of the pore-forming α subunit and the single TM-spanning β subunits. The auxiliary subunits have fundamental roles in the formation and localization of the channels, as their presence influences the biophysical properties of the channels reconstituted in heterologous expression systems [21]. These subunits are also essential for the channel function in vivo, as mutations in Ca_V β [22] or $\alpha 2\delta$ subunits [23] lead to severe disorders or lethality.

Unlike the composition of Na_Vs and Ca_Vs, the subunits of CATSPER channels are not well studied. Because all CATSPER pore-forming proteins (CATSPER1–4) are required for the functional alkalization-activated current, the channel pore is thought to be a tetramer of the four CATSPERs [11]. In addition, the channel complex contains the multiple TM-spanning protein CATSPERB (previously called CATSPERβ) [24]. Herein, we identify the novel single-TM protein CATSPERG associated with the CATSPER complex.

MATERIALS AND METHODS

Animals

All procedures described herein were reviewed and approved by the University of Pennsylvania Institutional Animal Care and Use Committee and were performed in accord with the *Guiding Principles in the Care and Use of Laboratory Animals* by the National Institutes of Health. The *Catsper1*$^{-/-}$ mouse was previously described [13]. *Tg(Catsper1/EGFP)1Dren* transgenic mice carry a transgene encoding a fusion protein between an HA-tagged green fluorescence protein (eGFP) and CATSPER1 (HA.EGFP.CATSPER1) in the *Catsper1*-null background [24]. Unlike the *Catsper1*-null mice, the male transgenic mice are fertile, suggesting that the fusion protein functionally replaced the wild-type CATSPER1.

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DYVLLELSID SCWVGSFYCP ILGFSATIHD AIATESTLFI RQNQLVYYFT GTYITLFDKS HGSSRWVRVL PSECIKRLCP VYASGNGSEY 360
VLALTTGKNE GYIHIGTITD GLVSFEMVPD GWSVCEKLPG KNCSIDWATY IADERNLLLL VKIDSGQFYL VNFNTEFKTL NILYKIPEFI 450
PEAKELDFLV LLDTVTYTNT PMTPKGLFFN TLNNMLYIWG NFILQSYNRE EFIFLADFPK ESTIKYMVNS FKGQMAVVTE NEEIWYFLEG 540
GYDVYQVVPS QGWETYHNLQ KMQKSSFHSE DESLVSLFFE DGKLFQLVYL FDVGKERLVK RLLPVGTLME YNLPKPFTVV NQGNYQAISF 630
THTCPFKEIH LIDVPKKHHA SRTESYVALP PLVSESLGFH NNNTLAVYQG LVYYLLWLHS KYDKPYADPV HDPTWRWWQH KTKDKDYFFY 720
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VLLNRNAVIN KIKVIDKKRC SEQGMIGRNI KKTSMMLKVL GAPGNCIQRT YLGGIIQGFK VVPIFIGCPP GKRLAFDVSY TIMHSEEINK 900
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KFYIMSHESP GVEWLCLENS PCYDIIPQSI YPPEFFFKLL VSNRGVDNST YCDYKLTFIV HIHGLPLSSK RTSFIVMVST SFFIALVVFY 1080
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                                                                    TM
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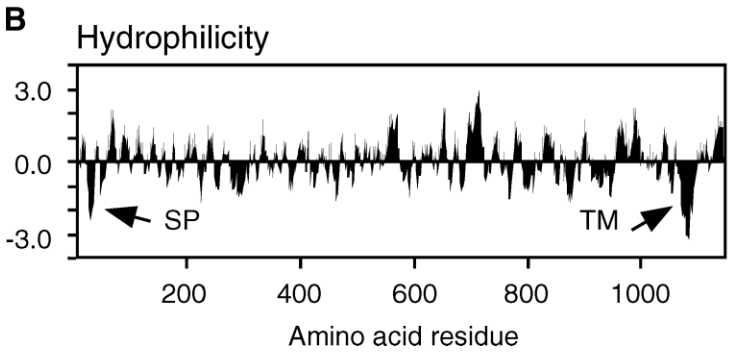
B



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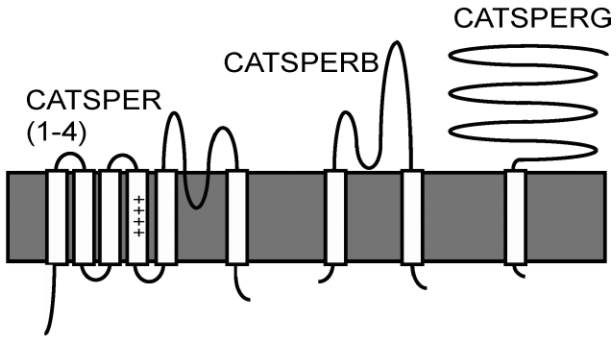
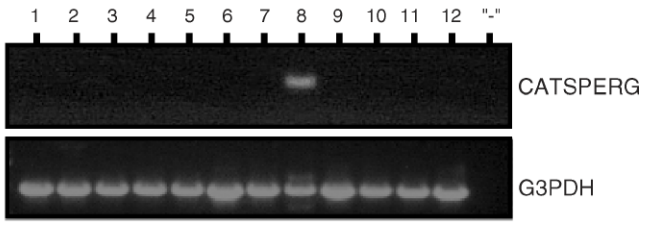


FIG. 1. CATSPERG. **A**) Predicted mouse CATSPERG protein sequences. Predicted signal peptide (SP) and TM domain are boxed. The five peptides identified by mass spectrometry are underlined (amino acids 66–77, 416–422, 439–445, 446–454, and 516–522). **B**) Hydrophilicity plot (window size, 11). **C**) A model depicting the composition of CATSPER channel complex and the putative membrane topology for CATSPERs (CATSPER1–4) [11], CATSPERB [24], and CATSPERG.

A



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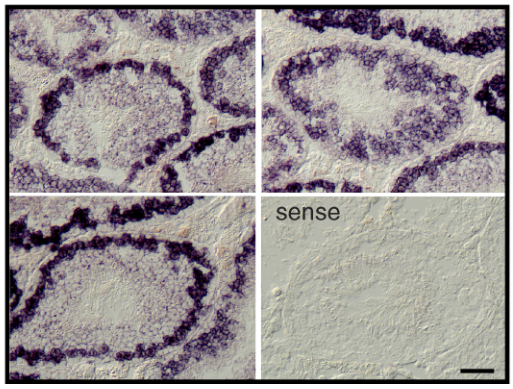

FIG. 2. Expression of *CATSPERG* mRNA. **A**) RT-PCR of CATSPERG (upper panel) and G3PDH (control [lower panel]) from 12 mouse cDNAs (lanes 1–12). Water served as negative control (lane "-"). Lane 1: heart; lane 2: brain; lane 3: spleen; lane 4: lung; lane 5: liver; lane 6: skeletal muscle; lane 7: kidney; lane 8: testis; lane 9: 7-day embryo; lane 10: 11-day embryo; lane 11: 15-day embryo; and lane 12: 17-day embryo. **B**) Representative fields of in situ hybridization in mouse testis using an antisense CATSPERG probe. Sense probe served as a background control (lower right panel). Bar = 10 μm.

Protein Purification and Identification

The CATSPER1 protein complex was purified from mouse testis as previously described [24]. Purified proteins were separated on SDS-PAGE gel, and specific protein bands were excised and subjected to protein identification using mass spectrometry. Five previously nonanalyzed CATSPERG peptides were identified in the band also containing CATSPERB (predicted to be 126 kDa, close to the 131 kDa of CATSPERG).

Cloning of CATSPERG

Novel peptide sequences from mass spectrometry were used to search databases to assemble putative open reading frames (ORFs). The whole CATSPERG ORF (GenBank accession number GQ225581) was amplified from mouse testis first-strand cDNA by PCR. To ensure that no mutations were incorporated in the PCR products, sequences were compared with genomic and cDNA expression sequence tag sequences; clones without unwanted mutations were used to assemble the full length into pcDNA3-based vector. The start of the ORF was determined by the presence of an in-frame stop codon in the 5′ untranslated region. Sequences of human, sea urchin, and *Ciona intestinalis* CATSPERG homologs were obtained by searching National Center for Biotechnology Information databases and the *C. intestinalis* cDNA databases [25].

Expression Analysis

To identify the overall expression pattern, CATSPERG RT-PCR was performed using a multiple-tissue mouse cDNA panel (Clontech, Palo Alto, CA). The forward and reverse primers had the following sequences: 5′-AGT CGA GTG GCT GTG CTT GGA GAA C-3′ and 5′-CTA TGC TGT CTT AGC TTG AAC ATT GTC C-3′, respectively. The RT-PCR amplification included 35 cycles of 20 sec at 94°C, 20 sec at 58°C, and 30 sec at 72°C. The mouse G3PDH was amplified as an input control for the cDNA panel kit, and the PCR samples in Figure 2A were drawn from the same reactions used in the study by Liu et al. [24]. To determine the specific localization of CATSPERG in the testis, a 1.4-kilobase single-strand digoxigenin-labeled RNA probe was

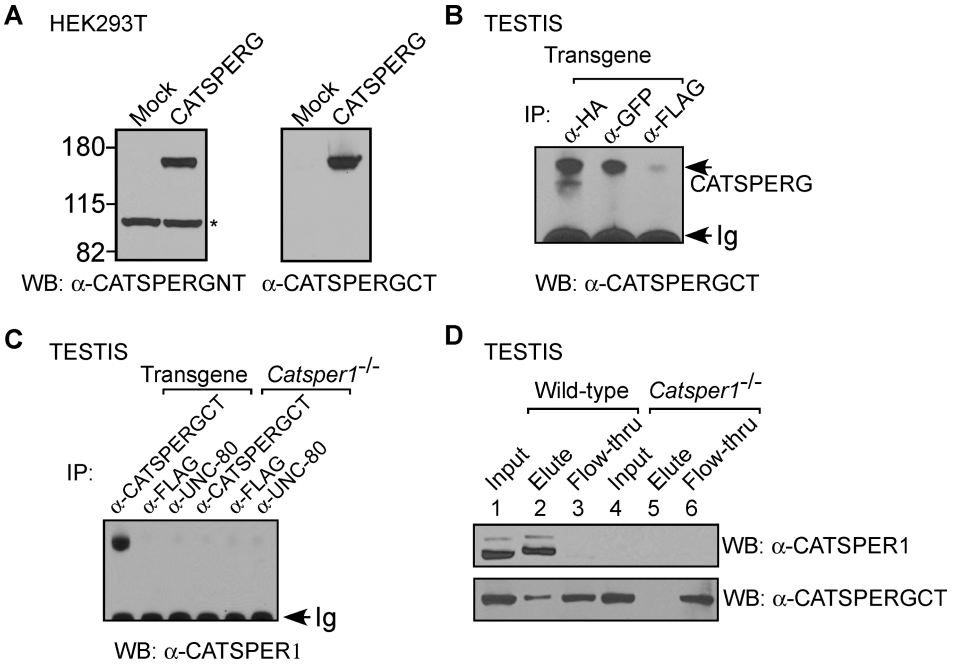

FIG. 3. Association between CATSPERG and CATSPER1 proteins. **A**) Antibody specificity. Total lysates from HEK293T cells transfected with CATSPERG cDNA or with empty vector (mock) were immunoblotted (WB) with two anti-CATSPERG antibodies (α-CATSPERGNT [left panel] and α-CATSPERGCT [right panel]). The asterisk in the left panel indicates a nonspecific band detected in both the mock- and CATSPERG-transfected cells. Molecular weights are in kilodalton (kDa). **B**) Association between CATSPERG and HA.EGFP.CATSPER1. Total testis membrane proteins prepared from *Tg(Catsper1/EGFP)1Dren* transgenic mice were immunoprecipitated (IP) with anti-HA, anti-GFP, or anti-FLAG (negative control) and blotted with anti-CATSPERGCT antibody. The lower bands are presumed immunoglobulins (Ig). A faint band at the position of CATSPER in lane α-FLAG is presumably a result of nonspecific binding. **C**) The reverse experiment using anti-CATSPERG antibody (anti-FLAG and anti-UNC-80 as negative control antibodies) for immunoprecipitation. Precipitations from *Catsper1*$^{-/-}$ testis membrane protein were used as a negative control for input. **D**) Binding of CATSPERG to the cobalt column via interaction with CATSPER1. Testis proteins from wild-type or *Catsper1*$^{-/-}$ mice were incubated with cobalt-conjugated agarose and washed. The flow through and final elution were probed with anti-CATSPER1 (upper) or anti-CATSPERGCT (lower).

synthesized with a T7 primer (starting at base 2068) and was used in in situ hybridization to testis sections (10 μm thick) as previously described [24].

Antibodies

Two polyclonal antibodies were developed by immunizing rabbits with keyhole limpit hemocyanin-conjugated peptides with the following sequences derived from mouse CATSPERG: QDHEPIDTVAKVFQC (for anti-CATSPERGNT [N-terminal amino acids 67–80]) and CRAGSKEDNVQAKTA (for anti-CATSPERGCT [C-terminal amino acids 1132–1145]). Antisera were affinity purified against the peptides linked to SulfoLink agarose (Pierce, Rockford, IL). Antibodies against CATSPER1 and UNC-80 (a neuronal ion channel-associated protein) were previously described [13, 26]. The following antibodies were purchased from commercial suppliers: anti-KCNU1 (mSLO3; NeuroMab, Davis, CA), anti-FLAG (Sigma, St. Louis, MO), anti-HA (Santa Cruz Biochemicals, Santa Cruz, CA), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (Pierce), and anti-GFP and Alexa 568-conjugated anti-rabbit IgG (Invitrogen, San Diego, CA).

Immunoprecipitation

Testis membrane protein was prepared as previously described [24]. About 500 μg of protein was solubilized in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.5% [w/v] deoxycholate, and 0.1% [w/v] SDS) supplemented with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN) at 4°C for 1 h. After a 30-min spin at ~21 000 × *g*, the supernatant was mixed with anti-HA, anti-GFP, or anti-FLAG (negative control) to precipitate HA.EGFP.CATSPER1 (or with anti-CATSPERGCT [anti-UNC-80 and anti-FLAG as negative controls] to precipitate CATSPERG). Following incubation at 4°C for 2 h, samples were mixed with 50 μl of RIPA buffer-equilibrated protein A agarose at 4°C for 2 h or overnight. After three washes (10 min each) with RIPA buffer, bound protein was eluted in 50 μl (for Fig. 3B) or 60 μl (for Fig. 3C) of lithium dodecyl sulfate (LDS) sample buffer, and 15 μl (for Fig. 3B) or 3 μl (for Fig. 3C) of the elution was analyzed using Western blot.

Cobalt Column Binding

Testis membrane protein (~1 mg) was solubilized with 1% Triton X-100 in 250 μl of binding buffer (50 mM Na_2HPO_4, 300 mM NaCl, and 1× protease inhibitor cocktail [pH 8.0]) at 4°C for 1 h. Soluble protein was added to 100 μl of buffer-equilibrated cobalt resin (Clontech) supplemented with 20 mM imidazole (pH 7.5) in 250 μl of binding buffer and mixed at 4°C for 2 h. Following a quick spinning of the protein-resin mixture, the supernatant (flow through) was collected and concentrated with a Microcon-50 ultrafiltration column (Millipore, Bedford, MA), and the cobalt resin was washed twice with binding buffer. Bound protein was eluted with 300 mM imidazole in binding buffer. Flow through and eluate were mixed with LDS loading buffer and heated for 15 min at 70°C before electrophoresis.

Western Blotting

SDS-PAGE was performed using the 4%–12% Bis-Tris gradient gels in MOPS-SDS running buffer (NuPAGE gels; Invitrogen) supplemented with NuPAGE antioxidant according to the manufacturer's instructions. Resolved proteins were transferred onto polyvinylidene difluoride membrane (Immuno-Blot; Bio-Rad, Cambridge, MA). The membrane was blocked with 5% nonfat dry milk in PBS with 0.1% Tween-20 and incubated with primary antibody at 4°C overnight. Anti-CATSPERGNT and anti-CATSPERGCT were used at ~0.3 μg/ml and ~0.65 μg/ml, respectively. Following incubation with HRP-labeled secondary antibody for 1 h at room temperature, the membrane was developed with SuperSignal ECL (Pierce).

Immunostaining

Because CATSPERG is only detected in testis, the anti-CATSPERGCT antibody was preabsorbed against cyanogen bromide-activated-Sepharose beads (Sigma-Aldrich, St. Louis, MO) conjugated with total protein from mouse brain, heart, and kidney to minimize nonspecificity. Sperm cells were spotted onto coverslips and fixed in cold acetone at −20°C for 2 min. After permeabilization with 0.2% Triton X-100 in PBS for 15 min, cells were blocked with 3% bovine serum albumin (BSA) in PBS for 30 min and

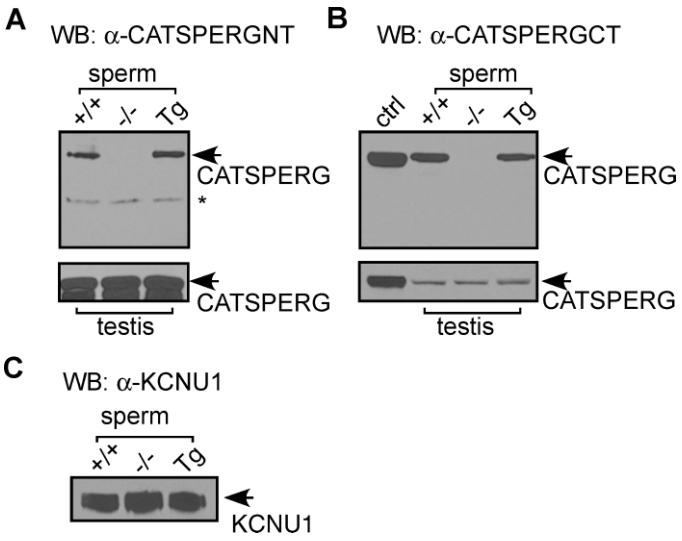

FIG. 4. Disruption of the *Catsper1* gene leads to a loss of CATSPERG protein but not a potassium channel protein in sperm. **A** and **B**) Total proteins from sperm (upper [from ~6×10^5 cells]) and membrane proteins from testis (lower [~80 μg]) prepared from wild-type (+/+), *Catsper1*$^{-/-}$ (-/-), and *Tg(Catsper1/EGFP)1Dren* transgenic (Tg) mice (in a *Catsper1*$^{-/-}$ background) were blotted with anti-CATSPERGNT (**A**) or anti-CATSPERGCT (**B**). The asterisk in **A** indicates a nonspecific band (see Fig. 3A). Protein lysate from CATSPERG cDNA-transfected HEK293T cells was loaded as molecular weight control (**B** [lane labeled "ctrl"]). **C**) Total sperm proteins probed with anti-KCNU1. WB, immunoblotted.

incubated with 0.65 μg/ml preabsorbed anti-CATSPERGCT in PBS containing 3% BSA at 4°C overnight. After three washes with 0.05% Triton X-100 in PBS, coverslips were incubated with Alexa 568-conjugated donkey anti-rabbit IgG secondary antibody (2.5 μg/ml) in PBS containing 10% normal goat sera at room temperature for 90 min, washed three times for 10 min each with 0.05% Triton X-100 in PBS, and mounted in antifade reagent (ProLong Gold; Invitrogen) for visualization with a Nikon (Natick, MA) fluorescence microscope with a 60× oil immersion lens. Wild-type and *Catsper1*$^{-/-}$ sperm were processed identically.

RESULTS

A Novel Single-TM Protein in the CATSPER Complex

We previously purified the CATSPER1-containing protein complex from mouse testis using transgenic mice expressing an HA- and eGFP-tagged CATSPER1 protein (HA.EGFP. CATSPER1) in the *Catsper1*-null background [24]. When separated on SDS-PAGE gel, the purified protein complex has two specific bands in addition to the CATSPER1-containing one. One of the bands was identified as HSPA1B, a testis-specific heat shock protein. Twelve peptides from the other band at ~130 kDa were identified as CATSPERB (predicted molecular weight, 126 kDa) [24]. Further protein purification preparation and mass spectrometry analysis of the ~130-kDa band revealed five additional peptides that do not match CATSPERB. Using these five peptide sequences, we cloned a novel protein, CATSPERG. The mouse CATSPERG is predicted to be 1145 amino acids, with a predicted molecular weight of 131 kDa and a isoelectric point (pI) of 6.7 (Fig. 1A). The CATSPERG sequence is not similar to that of any other protein with known function. A hydrophilicity plot predicts one signal peptide, a large extracellular domain (~1000 amino acids), a TM-spanning domain, and a short intracellular tail (58 amino acids) (Fig. 1, B and C). The predicted TM topology is similar to that of the α2δ subunit of Ca_V channels and the β subunits of Na_Vs [20].

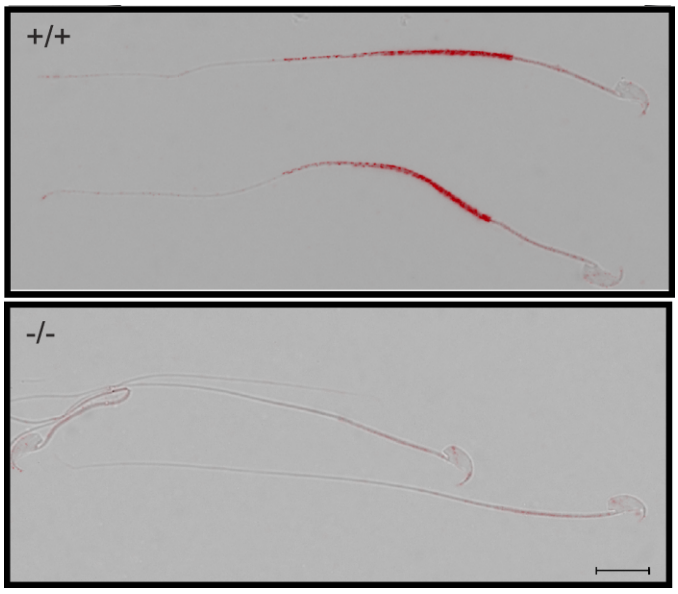

FIG. 5. Localization of CATSPERG in the sperm principal piece. Immunofluorescence staining with anti-CATSPERGCT antibody in wild-type (upper [+/+]) and *Catsper1*$^{-/-}$ (lower [-/-]) sperm. Immunofluorescence (in red) localizes specifically to the principal piece. Bar = 10 μm.

CATSPERG homologs are present in the genomes of all animals that have CATSPER channels, including humans, mice, *C. intestinalis*, and sea urchins, but are absent from the genomes of animals without CATSPER channels, including *Drosophila melanogaster* and *Caenorhabditis elegans*. Like other CATSPER complex proteins, CATSPERG has a low sequence identity (55%) between human and mouse.

CATSPERG Is Expressed in Testis

We used RT-PCR to determine when and where *CATSPERG* is expressed. *CATSPERG* mRNA was only detected in testis (Fig. 2A). The expression pattern is highly similar to that of CATSPER1–4 and CATSPERB [11, 13, 24, 27, 28]. To determine the expression within testis, we performed in situ hybridization with labeled RNAs derived from CATSPERG cDNA. Sense probe as a specificity control did not detect significant signal; antisense probe detected expression in the seminiferous tubules but not in the interstitial cells. Within the seminiferous tubules, CATSPERG was detected in spermatocytes and spermatids (Fig. 2B). Further experiments are needed to compare the expression time windows of *CATSPER1–4*, *CATSPERB*, and *CATSPERG*.

Association of CATSPERG and CATSPER Proteins in Testis

We developed two polyclonal antibodies against the amino-termini and carboxy-termini of CATSPERG (α-CATSPERGNT and α-CATSPERGCT, respectively). Both antibodies specifically recognized CATSPERG protein in CATSPERG cDNA-transfected HEK293T cells (Fig. 3A). Proteins immunoprecipitated from the HA.EGFP.CATSPER1 transgenic testis with anti-HA or anti-GFP antibodies were recognized by the antibody against CATSPERG (Fig. 3B). Conversely, immunoprecipitation with an anti-CATSPERG antibody also precipitated CATSPER1 as detected by an anti-CATSPER1 antibody (Fig. 3C). These data confirmed the association between CATSPER1 and CATSPERG in testis.

CATSPER1 protein binds to a cobalt column, presumably because of the protein's histidine-rich N-terminus (Fig. 3D, upper panel) [24]. CATSPERG, which has no histidine-rich region, could be detected from proteins eluted from cobalt columns incubated with wild-type mouse testis protein but not from those incubated with *Catsper1*$^{-/-}$ testis protein (Fig. 3D), suggesting a binding of CATSPERG to the cobalt column via its association with CATSPER1. In the cobalt column flow through, CATSPER1 protein was largely undetectable (Fig. 3D, upper panel, lane 3), whereas CATSPERG could be detected (Fig. 3D, lower panel, lane 3), suggesting that not all CATSPERG protein is associated with CATSPER1 in testis.

Dependence of CATSPERG Protein on CATSPER1 in Sperm

In wild-type mouse sperm, both of the CATSPERG antibodies recognized a protein that migrated at the same position as that from CATSPERG cDNA-transfected HEK293T cells (Fig. 4, A and B). In the *Catsper1*$^{-/-}$ sperm, however, CATSPERG was undetectable; CATSPERG protein was restored in the *Catsper1*$^{-/-}$ sperm rescued with the transgene *Tg(Catsper1/EGFP)1Dren* (Fig. 4). Unlike in sperm, CATSPERG protein levels in testis were similar between wild type, *Catsper1*$^{-/-}$, and transgenic (Fig. 4, A and B). These data suggest that the presence of CATSPERG protein in sperm, but not in testis, depends on the presence of CATSPER1 protein.

The loss of CATSPERG protein (Fig. 4, A and B) (as well as another CATSPER channel pore-forming protein, CATSPER2 [29], and the CATSPER-associated protein CATSPERB [24]) in *Catsper1*$^{-/-}$ sperm raised the suspicion that the disruption of the *Catsper1* gene in the knockout leads to nonspecific degradation or instability of sperm proteins. This effect, however, appears to be specific for CATSPER-associated proteins, as KCNU1 (a testis-specific protein [30] that presumably forms the alkalization-activated potassium channel K_{Sper} [31]) was not disrupted in the *Catsper1*$^{-/-}$ sperm (Fig. 4C). The intact KCNU1 protein in *Catsper1*$^{-/-}$ sperm is consistent with the detection of the K_{Sper} channel current in the *Catsper1*$^{-/-}$ mutant sperm [31]. These data suggest that only the CATSPER-related pore-forming subunits and auxiliary proteins are CATSPER1 dependent.

Localization of CATSPERG Protein in Sperm Principal Piece

To determine the subcellular localization of CATSPERG, we performed immunofluorescence staining with wild-type and *Catsper1*$^{-/-}$ sperm. A CATSPERG antibody recognized signals localized in the principal piece (Fig. 5). Consistent with the absence of protein in the *Catsper1*$^{-/-}$ sperm, the antibody detected minimal signals in the mutant sperm.

DISCUSSION

Herein, we describe CATSPERG, a novel single TM-spanning protein associated with CATSPER. We provide three lines of evidence supporting CATSPERG as a component of the CATSPER complex: 1) CATSPERG copurified with CATSPER1, 2) it colocalized with CATSPER proteins, and 3) it was undetectable in the *Catsper1*$^{-/-}$ sperm and was restored with an HA.EGFP.CATSPER1 protein in the transgenic mice. In addition, CATSPERG shares many common features with other CATSPER-related proteins. For example, all four CATSPERs (CATSPER1–4), CATSPERB, and CATSPERG are expressed exclusively in testis; the proteins are strikingly localized to the sperm principal piece [11, 13, 24, 28] (see also the study by Jin et al. [14]), consistent with the detection of functional ionic currents [18] and Ca^{2+} influx [17] mediated by the CATSPER channels in the same sperm subcellular region. Furthermore, they are all found in the same unique set of animals but not in *D. melanogaster* and *C. elegans* [32]. Like many other fertilization-specific proteins, the CATSPER-related ones between human and mouse have a much lower sequence identity (~55%) than those most other proteins [32, 33].

The CATSPER protein complex consists at least three TM proteins: the pore-forming six-TM CATSPER1–4, a predicted two-TM CATSPERB [24], and the single-TM CATSPERG described herein. This structure is comparable to that of other ion channels such as Ca_Vs, Na_Vs, and K_Vs. In several Ca_Vs, the auxiliary subunits control the trafficking of channel complex and are required for a robust functional reconstitution of proper ion channel currents in heterologous systems [21]. Further functional reconstitution studies will be needed to test whether CATSPERG functions as an auxiliary subunit of the CATSPER channel. In preliminary experiments, we have coexpressed CATSPERs with CATSPERB and CATSPERG but have not detected functional channel currents (Ren et al., unpublished results). The inability to reconstitute a functional CATSPER channel remains unclear. In the protein purification, we did not detect other major proteins in the CATSPER1-containing complex [24]. Other proteins might have a role in the complex, but they may have a weak association with CATSPER1 that does not survive the purification with detergents [11]. Alternatively, the necessary subunits may interact with CATSPER only in sperm and not in testis cells, which are the major source of the purified CATSPER protein complex.

CATSPER channels can be directly activated by intracellular alkalization [18]. However, the channels might also be activated by other biological stimuli. Before fertilization, sperm cells interact with many other cell types within the seminiferous tubules, the male and female reproduction tracts, the cumulus cells around the egg, and finally the egg coat and membrane. In Na_V channels, the extracellular domains of the single TM-spanning β subunits modulate the Na_V's biophysical properties and function as adhesion molecules in the interaction between neurons and glia [34]. It will be relevant to determine whether the large extracellular domain of CATSPERG (~1000 amino acids) has similar roles in mediating the interaction between sperm and other cells and/or in coupling of environmental stimuli to channel opening.

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