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Molecular cloning and characterization of SCaMPER, a sphingolipid Ca^{2+} release-mediating protein from endoplasmic reticulum

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ABSTRACT **Release of Ca^{2+} stored in endoplasmic reticulum is a ubiquitous mechanism involved in cellular signal transduction, proliferation, and apoptosis. Recently, sphingolipid metabolites have been recognized as mediators of intracellular Ca^{2+} release, through their action at a previously undescribed intracellular Ca^{2+} channel. Here we describe the molecular cloning and characterization of a protein that causes the expression of sphingosyl-phosphocholine-mediated Ca^{2+} release when its complementary RNA is injected into *Xenopus* oocytes. SCaMPER (for sphingolipid Ca^{2+} release-mediating protein of endoplasmic reticulum) is an 181 amino acid protein with two putative membrane-spanning domains. SCaMPER is incorporated into microsomes upon expression in Sf9 cells or after translation *in vitro*. It mediates Ca^{2+} release at 4°C as well as 22°C, consistent with having ion channel function. The EC_{50} for Ca^{2+} release from *Xenopus* oocytes is 40 μM, similar to sphingosyl-phosphocholine-mediated Ca^{2+} release from permeabilized mammalian cells. Because Ca^{2+} release is not blocked by ryanodine or La^{3+}, the activity described here is distinct from the Ca^{2+} release activity of the ryanodine receptor and the inositol 1,4,5-trisphosphate receptor. The properties of SCaMPER are identical to those of the sphingolipid-gated Ca^{2+} channel that we have previously described. These findings suggest that SCaMPER is a sphingolipid-gated Ca^{2+}-permeable channel and support its role as a mediator of this pathway for intracellular Ca^{2+} signal transduction.**

Sphingolipids are emerging as key regulators of cellular metabolism, proliferation, and apoptosis (1, 2). One of the cellular actions of sphingolipids is the release of Ca^{2+} from intracellular stores (3, 4). Sphingosine, sphingosine phosphate, and sphingosyl-phosphocholine (SPC) all mediate release of Ca^{2+} from intracellular stores. Until recently, the mechanism by which sphingolipids could release Ca^{2+} from intracellular stores was unknown. We have described the electrophysiological and biophysical properties of a sphingolipid-gated intracellular Ca^{2+}-permeable channel (5, 6). The sphingolipid-gated Ca^{2+}-permeable channel is unlike other known channels. It is not blocked by La^{3+}, a prototypical blocker of Ca^{2+} selective channels, nor is it blocked by heparin, nifedipine, ω-conotoxin, or Ni^{2+}, all selective blockers of specific classes of voltage-gated and ligand-gated intracellular and plasma membrane Ca^{2+} channels. We developed an assay to examine expression of sphingolipid-gated Ca^{2+} release from the intracellular stores of *Xenopus* oocytes (6). With this, we demonstrated that the mRNA encoding the intracellular sphingolipid-gated Ca^{2+} release activity is ≈1.8 kb, much smaller than the ≈16-kb ryanodine receptor message (7), or the ≈9-kb message for the inositol 1,4,5-triphosphate ($InsP_3$) receptor (8). To further characterize the molecular mechanism by which sphingolipids release Ca^{2+} from intracellular stores, we used sib selection methods and expression of functional activity in *Xenopus* oocytes to identify the cDNA encoding SCaMPER (sphingolipid Ca^{2+} release-mediating protein of endoplasmic reticulum).*

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METHODS

Cloning by Expression in *Xenopus* Oocytes. We used a cDNA library derived from MDCK (canine kidney) mRNA and cloned in pCDNA1 [A kind gift of E. Peralta, Harvard University (9)]. The library bears ≈200,000 independent recombinants and was divided into 11 equal pools. Complementary RNA (cRNA) was synthesized by using the T7 RNA polymerase (10) (mCap RNA synthesis kit, Stratagene, and mMessage Machine, Ambion). Preparation and microinjection of stage 6 *Xenopus laevis* oocytes were performed as described previously (6). Approximately 50 ng of cRNA was injected into each oocyte. The oocytes were incubated for 2 days at 19°C. Oocytes were then permeabilized with 10 μM digitonin and subjected to the Ca^{2+} efflux assay as described previously (6). Most of the studies were performed with fluo-3 (Molecular Probes) as the Ca^{2+} indicator. Studies with La^{3+} were performed by using $^{45}Ca^{2+}$ (La^{3+} has a high affinity for fluo-3, and its binding results in an intense fluorescence).

Dideoxynucleotide sequencing of plasmid DNA was performed using Sequenase 2.0 (United States Biochemical) or Ladderman (Takara). Northern blotting was performed by standard methods (11).

Translation *in vitro* was performed with reticulocyte lysates (Novagen) in the absence and presence of canine microsomes (Promega).

Construction of SCaMPER-ORF. SCaMPER-ORF was constructed by digesting the full-length clone with *Hin*dIII and *Xba* I. The full-length cDNA was then ligated into a similarly digested pBluescript KS vector, placing the 5′ end of the open reading frame (ORF) just downstream of the T3 promoter. This construct was further digested with *Hin*dIII and *Nde* I, to remove the 5′ untranslated region (UTR). Following blunt-end religation, the construct was further digested with *Sma* I and *Xba* I to remove all but 250 bases of the 3′ UTR. Sequence and orientation were then confirmed by restriction digestion and dideoxy-nucleotide sequencing.

Abbreviations: SPC, sphingosyl-phosphocholine; $InsP_3$, inositol 1,4,5-trisphosphate; cRNA, complementary RNA; ORF, open reading frame; UTR, untranslated region.
C.M. and S.H.K. contributed equally to this work.
*The sequence reported in this paper has been deposited in the GenBank data base (accession no. U33628).

Construction of SCaMPER-Bac. The 250-base 3′ UTR of SCaMPER-ORF was eliminated by using primer extension PCR, and 8 amino acids constituting the FLAG epitope (DYKDDDDK) were added to the carboxyl terminus of SCaMPER. This construct was subcloned in the insect virus *Autographa californica* pVL 1393 (Baculogold, Pharmingen) to create SCaMPER-Bac. Following infection of Sf9 insect cells with recombinant virus, expression of SCaMPER was confirmed by Western blotting with anti-FLAG antibody (IBI-Kodak). Subcellular localization was confirmed by differential centrifugation (12) and Western blotting.

A similar construct was prepared by subcloning the FLAG-tagged ORF in the mammalian expression vector pME18S (13). This construct was transiently transfected into both Cos cells and 293 cells. Expression of epitope-tagged SCaMPER was not detected in either case.

RESULTS

To further characterize the mechanism by which sphingolipids could mediate the release of Ca^{2+} from intracellular Ca^{2+} stores, we cloned a cDNA which confers sphingolipid sensitivity to *Xenopus* oocytes. Using methods we have described previously (6), we screened a cDNA expression library (9) prepared from MDCK cells, a cell line which we had previously observed to express sphingolipid-mediated Ca^{2+} release (unpublished observations). Following eight rounds of sib selection, a unique 1869-nt cDNA was obtained (Fig. 1). This cDNA bears an ORF consisting of 181 amino acids. The calculated molecular mass of SCaMPER is 20,061 Da. Hydrophilicity (Kyte–Doolittle) and Chou–Fasman analysis reveal two potential transmembrane domains (Fig. 2). The sequence bears a single N-glycosylation consensus site, at amino acid 70. This

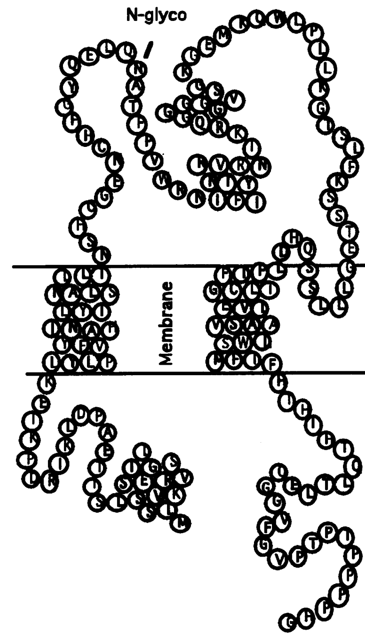

FIG. 2. Hypothesized secondary structure for SCaMPER: Snake diagram based on Kyte–Doolittle hydrophilicity and Chou–Fasman rules. The single putative N-glycosylation site suggests that the region between the two transmembrane domains is lumenal.

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1    CAAAATTTCC TTTTCAGTCA TGCTTTTAAG ATACTTTTTC TAAAAAGATT TATTTCCAGA GTGTTCCCAT GTTGGTTTAA AATTCACTGT TTTCAACATT
101  TTAGAGCAAT ACCTCATCTA CTTGATTTTG TGTCTGCTGA AGTCTTCGGT TGCTGCCAGG CGGCACTTGA CCCTGGGGTT GCACCTGCAG ACNCTGAAAT
201  GGGTAAAGGC ACAAGCATGA GTAAACTATG ATTCCAGATA CATTTTTTAT AAGTATGAAG ACATTATCCC AAGGCATCAC TGTTTAGAGG TTCAGAACTT
301  AAGCAGAAAA AGAAACCCTT CCTACAGTAA GGAACAAAGG TTCTAATGAC ATTTCTTCTA ATTCCTCTAT AATTTCCAGG ATTCATCATA TGTTAAAAGT
401  GAGCAGGGTC TCAAGTGAAG GTTTAATATC ACTTTCTATC ACTGAGGCAC CTGATCTTAA GATCAGGGAT CCTAAGATAG AGAAACTCTA CCTTCCAGTT
501  TTTTATTTAA ATGCACACAT CTACTTAAAT GCACTCAGTA CTCTCCTGAA CTCTCATTGT GGCGAGAACT GTTTTCATGG TTATGAACAA TTACAGAATG
601  CCACTTTTCC AGTTTGGAGA AATATATTCA TTTATATAAA CAGGGTCAGG AACATCAAGA GGCAAGGAGG AGGGGGTGGT GTGAGTGGGA AAGGTGAGAT
701  GAAGCAGTGC CTTCCTCTCT TAAAAGGTAC TTCTCTTTTC AAATCCTCAA CTGAAGGCCT ATTGCTCTCC AGCCAGATCT TTCTTGACCA CTGTGGCTTT
801  GAAGTCCTTA TCGCTTCCGT TTCTTGGCTG GCCATGCTTT TCATTTTTCA CACACACATA CACACACAGC TGACCCTTGA ACAAGGTGGG GTGTTTGGGG
901  TGCCGACGCC GACCCCTCCC CCTCCCCATG GTTGAAAATC TGCATATAAC TTTTGACTCC TCCCAAAATT TAACTACTAA TAGCCTACTG CTGACCAAAG
1001 GCCTTACCAA TAACATAAAC AGTCGATTAA CAAGTATTTT GTATATTATA TGTATCATAC ACTGTGTTCT TACAGTAAAG TAAGCTAGAG CAGAAAATGT
1101 TAAGAAAATC GTAAGACAAG GGCAGGCCAG GTGGCTCAGC AGTTTAGCGC CACCTTCAGC CCAGGACTGA TCCTGGAGAC CCGGGATGGA GTCCCATGTC
1201 GGGCTCCCTG CATGGAGCCT GCTTCTCCCT CTGCCTGTGT CTCTCTCATG AATAAATACA TAAAATCTTA AAAAAAAAAA AAAAAAAAAA AAAGGAAATC
1301 GTAAGACAGA GAAAATACGT TTACATTACT GTATTGTATT TATTGGAAAA AAACCCACCT GTAAGTGGAC CAGCACAGTT CAAACCCATA TTGTTCAAGG
1401 GTCATCTGTA ATGTCTTGCC TTCCCTGATA GATCACAGCT CCTGTGTCTT ACAGGTCTCT TCCCTGTACC CAAGACAGCA CCTAGCCTGG GCCTTGACTA
1501 CAACAGCTAC TGATGCACCC ACTGATTCAA GTTATTTTAT ACCTACCACA TGTCAAAGAC CAGGGATGCA GCAGATCCCA TGCCTGCCCT TACACTTTAT
1601 TTTTATTCTC TGATAGGTAA TAGCGAAGAA CCATGCAATG GCACATTTTC TCACTTAGCA TTTTTCTCAT TCAGTCCTTT CAACTCTTTG AGGGTGTGTG
1701 TGTGTACTAC TATCAACCCC ATTTCTGGAA GCAAAAAGTG GGATTTAGAA AGTCAACAGT GCTGGTTGCA GTGGAACCTG TCTTAGACCA GGTCTCCTAT
1801 AATTCCACCG ATCTAGACCT TAATTAACCA TTGTGATTTA CTGGCACGGC AGCCGCGGCA GGTACACTC

1   MLKVSRVSSE GLISLSITEA PDLKIRDPKI EKLYLPVFYL NAHIYLNALS
51  TLLNSHCGEN CFHGYEQLQN ATFPVWRNIF IYINRVRNIK RQGGGGGVSG
101 KGEMKQCLPL LKGTSLFKSS TEGLLLSSQI FLDHCGFEVL IASVSWLAML
151 FIFHIHIHTQ LTLEQGGVFG VPTPTPPPPH G
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FIG. 1. Complete cDNA sequence (*Upper*) and deduced amino acid sequence (*Lower*) of the ORF encoding SCaMPER. The underlined nucleic acid sequence (nucleotides 389–932) is the ORF.

suggests that the peptide loop residing between the two transmembrane domains is intralumenal, and that the N amino- and carboxyl-termini are cytosolic.

Following *in-vitro* transcription and injection into *Xenopus* oocytes, both the full-length cDNA and the subcloned SCaMPER-ORF conferred the desired phenotype of rapid SPC-induced Ca^{2+} release to digitonin-permeabilized *Xenopus* oocytes (Fig. 3*A*). A total of 10 sets of oocytes, injected with cRNA from three separate preparations of RNA, gave essentially the same result. The mean ± SEM of Ca^{2+} release was 35% ± 8% of the total ionophore-releasable Ca^{2+}. We examined several types of controls. The first was oocytes injected with diethylpyrocarbonate (DEPC)-treated water. Mobilization of Ca^{2+} in response to SPC was rarely observed, and it was never more than 10% of the total ionophore-releasable Ca^{2+} (mean of Ca^{2+} release by 80 μM SPC in six water-injected control runs was 3% ± 5%). We also injected cRNA produced from five randomly selected clones bearing inserts 1–2 kb in size, as well as a commercially available 7.5-kb marker RNA (GIBCO). Ca^{2+} was not mobilized by addition of SPC to any of these controls. Ca^{2+} release continued at a rate equivalent to the background leak of Ca^{2+}. Finally, injection of antisense SCaMPER cRNA also does not result in expression of Ca^{2+} release in response to SPC.

We next examined the dose response of SCaMPER cRNA-injected oocytes to SPC. The dose response for Ca^{2+} mobilization is similar to that we previously observed for SPC-induced Ca^{2+} release from permeabilized cells (5, 6), with the half-maximal release rate occurring at 30–40 μM SPC, and the maximal rate being reached at 60 mM (Fig. 3*B*). Ca^{2+} release is specific for SPC. Sphingosine at similar concentrations does not cause Ca^{2+} release (not shown).

We next examined a panel of potential agonists, inhibitors, and incubation conditions to define the pharmacologic and physiologic profile of SCaMPER. Sphingosine 1-phosphate, solubilized in saponin at 0.1 mg/ml, at apparent concentrations of up to 50 μM does not cause release of Ca^{2+} from permeabilized cells (data not shown). Ca^{2+} release was not blocked by La^{3+}, a pore blocker of many Ca^{2+}-selective ion channels. Ryanodine at 100 μM neither causes mobilization of Ca^{2+} from oocytes nor blocks SPC-mediated Ca^{2+} release from SCaMPER-injected oocytes (data not shown). Heparin, a competitive antagonist of InsP_3-gated Ca^{2+} release, also does not block release of Ca^{2+} in response to 80 μM SPC. Ca^{2+} release was observed at 4°C as well as at 22°C, thus favoring a channel function rather than a metabolic function for SCaMPER. Ca^{2+} release occurs only from digitonin-permeabilized oocytes, indicating that the site of action of SPC is intracellular. Ca^{2+} release in response to SPC is not observed in oocytes incubated with cycloheximide at 10 μg/ml for 2 days after injection of SCaMPER cRNA, indicating that protein synthesis is required for expression of SPC-induced Ca^{2+} release. Ca^{2+} release is also absent from water-injected oocytes incubated with cycloheximide at 10 μg/ml for the 2 days following injection (data not shown).

In-vitro translation of both the full-length cDNA and the subcloned ORF both resulted in a protein product of ≈25 kDa. This discrepancy with the predicted mass could be the result of the highly basic nature (pI = 9.13) predicted from the amino acid sequence. We could not obtain expression of FLAG-labeled SCaMPER in either Cos cells or 293 cells. We did obtain excellent expression in Sf9 cells after infection with SCaMPER-Bac, a construct bearing only the open reading from of SCaMPER with the FLAG epitope at its 3′ terminus. As seen in Fig. 4, SCaMPER-Bac is expressed and retained in a microsomal fraction. Translation of SCaMPER-ORF cRNA in the presence of canine microsomes also results in the incorporation of all newly synthesized protein into the membrane-bound fraction (data not shown).

We examined expression of SCaMPER by Using both antisense RNA probes and randomly primed restriction endonuclease-digested fragments. A Northern blot of MDCK poly(A)$^+$ RNA (Fig. 5) had a band at 1.8 kb, confirming that the cDNA cloned represents the full-length message. A tissue Northern blot revealed very low expression of SCaMPER in canine liver, kidney, heart, pancreas, and spleen (not shown).

DISCUSSION

Ca^{2+} release and sphingolipid turnover have been observed to occur simultaneously following exposure of cells to ionizing radiation (14, 15), tumor necrosis factor α (16, 17), and platelet-derived growth factor BB (18). Apoptotic signaling requires the release of Ca^{2+} from intracellular Ca^{2+} stores (19) and the activation of sphingomyelin turnover (20). In *Saccharomyces cerevisiae*, intact spingolipid metabolism is required for proper Ca^{2+} homeostasis (21). Inhibitors of sphingolipid-mediated Ca^{2+} release have not been identified, and thus the

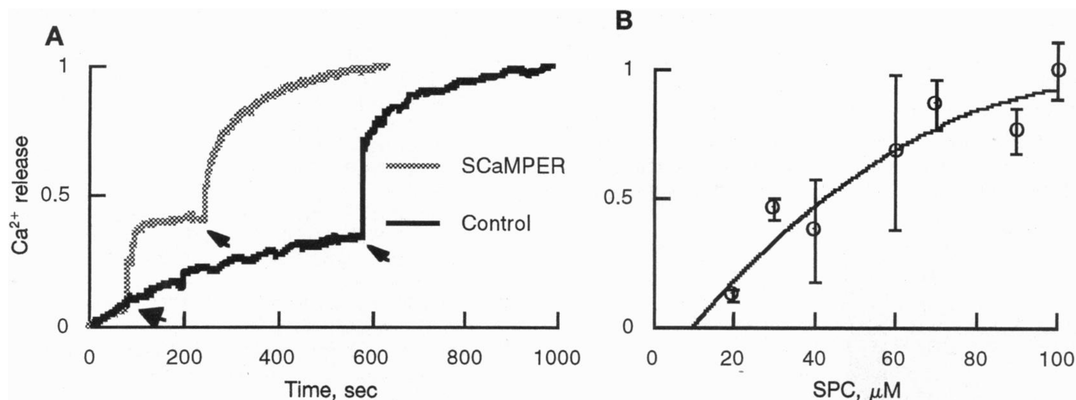

FIG. 3. Ca^{2+} release from permeabilized *Xenopus* oocytes after injection of cRNA encoding SCaMPER-ORF. Ca^{2+} efflux assay from permeabilized oocytes was performed exactly as described previously with "Ca^{2+} sponged" reagents (6), using 1 μM fluo-3, 0.4 mM ATP, and 2 mM $MgCl_2$. All assays were performed with three oocytes in the cuvette, at 22°C. (*A*) Time course. Thick arrow, addition of 80 μM SPC to both the control (injected with diethyl pyrocarbonate-treated water) and the SCaMPER-ORF cRNA-injected oocytes. Upon addition of SPC, the SCaMPER-injected oocytes demonstrate a burst of Ca^{2+} release, whereas the control-injected oocytes show only a continued baseline leak of Ca^{2+}. Thin arrows, addition of 10 μM Ca^{2+} ionophore A23187. The graph is normalized to display data as a fraction of the ionophore-releasable Ca^{2+}. The data shown are representative of 10 separate experiments. For these experiments Ca^{2+} release in response to SPC is 35% ± 8% of the ionophore-releasable Ca^{2+}. (*B*) Dose response to SPC of SCaMPER cRNA-injected permeabilized oocytes. Representative data from a series of SPC dose–response experiments performed as described for *A*, using the indicated SPC concentrations are shown. Ca^{2+} release is normalized relative to maximal SPC-induced release. Each point represents the mean ± SEM of four determinations.

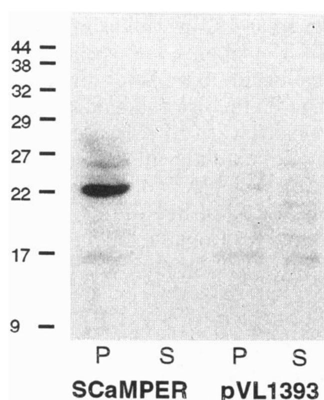

FIG. 4. SCaMPER is localized in microsomes of Sf9 cells. Sf9 cells were infected with either control baculovirus (pVL1393) or SCaMPER-Bac. Cells were incubated for 72 hr at 27°C and harvested and separated into pellet (P) and supernatant (S) fractions as described (12). Each lane of the SDS-polyacrylamide gel was loaded with approximately 70 ng of protein. The FLAG epitope was detected by enhanced chemiluminescence. Molecular sizes are expressed in kDa.

relationship of sphingolipid-mediated Ca^{2+} release and the Ca^{2+} response associated with these stimuli remains unknown. Furthermore, the mechanisms by which Ca^{2+} release could be mediated by sphingolipids remained undefined.

In this report we describe the molecular cloning by expression of SCaMPER, a 181-amino acid protein which mediates sphingolipid-gated Ca^{2+} release from intracellular stores. SCaMPER is structurally and functionally unique among known proteins. The sequence encoded by the ORF has no homology to known proteins. SCaMPER thus appears to be a representative of a new class of proteins capable of mediating sphingolipid-gated Ca^{2+} release.

SCaMPER may be the sphingolipid-gated channel we have described previously (5). SCaMPER is incorporated into microsomes, the location of the hormone-sensitive intracellular Ca^{2+} stores. Because Ca^{2+} release occurs at 4°C as well as at 22°C, it is not likely that SCaMPER is metabolizing SPC into another ligand which in turn mediates Ca^{2+} release. SCaMPER is not activated by sphinosgine, but is activated by SPC. Finally, like sphingolipid-mediated Ca^{2+} release is permeabilized cells, SCaMPER-mediated Ca^{2+} release is not blocked by La^{3+} or by ryanodine. Because SPC does not cause Ca^{2+} release from permeabilized control oocytes, by definition SPC is not causing Ca^{2+} release through a "nonspecific" detergent effect. While the data presented here are consistant with SCaMPER's being a novel ion channel, we cannot formally exclude the possibility that it functions as a regulatory protein of another as-yet-uncharacterized Ca^{2+}-permeable intracellular ion channel. Proof of its function as an ion channel awaits

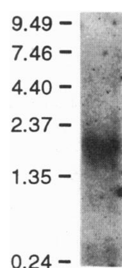

FIG. 5. Northern blot of RNA derived from MDCK cells. A 9-μg sample of poly(A)$^+$ RNA prepared from MDCK cells was electrophoresed through a 1.2% agarose gel with formaldehyde and transferred to a Nitran filter. The filter was then probed with a 300-bp fragment (*Bam*HI/*Bgl* II) from the ORF. The blot was exposed for 1 week. Molecular sizes are expressed in kilobases.

its high-level expression and incorporation into liposomes or planar lipid bilayers.

If SCaMPER is an ion channel, it is another member of the growing family of ion channels with two membrane-spanning domains. The amiloride-sensitive Na^+ channel has two transmembrane domains (22), as does the renal K^+-conducting inward rectifier (23, 24). Ductin, a 16-kDa constituent of gap junctions, is composed of a duplicated motif each bearing two transmembrane domains like SCaMPER. Ductin can form Na^+ channels (25). In contrast, the known ligand-gated Ca^{2+}-selective channels, such as the ryanodine receptor (7) and InsP_3 receptor (8), have six putative membrane-spanning domains. SCaMPER provides an additional reagent with which to study the biology of sphingolipid-mediated Ca^{2+} release in these and other systems, and it provides a molecular tool with which to probe the biologic function of this newly described signaling pathway.

Note Added in Proof. Due to restructuring at Duke University Medical Center, the laboratories of L.A.K. and J.S.A. are being closed. These authors thank their colleagues for their camaraderie and support over many years and thank the National Institutes of Health for its support of the early parts of this work.

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