Re-evaluation of primary structure, topology, and localization of Scamper, a putative intracellular Ca^{2+} channel activated by sphingosylphosphocholine

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Naturally occurring sphingoid molecules control vital functions of the cell through their interaction with specific receptors. Proliferation, differentiation and programmed death result in fact from a fine balance of signals, among which sphingosine and structurally related molecules play fundamental roles, acting as either first or second messengers. The corresponding receptors need to be identified in order that the role of sphingoid molecules can be established. Among them, several G-protein-coupled receptors specific for sphingosine 1-phosphate, sphingosylphosphocholine, or both, have already been investigated. In contrast, the identification of the postulated intracellular receptors has been problematical. In the present study we re-evaluated the molecular characterization of Scamper, the first proposed intracellular receptor for sphingosylphosphocholine [Mao, Kim, Almenoff, Rudner, Kearney and Kindman (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 1993–1996] and commonly believed to be a Ca^{2+} channel of the endoplasmic reticulum (the name 'SCaMPER' used by Mao et al. being derived from '**s**phingolipid **Ca**$^{2+}$-release-**m**ediating protein of the **e**ndoplasmic **r**eticulum'). In contrast with what has been believed hitherto, our primary-structure and overexpression experiments indicate that Scamper is a 110-amino-acid protein spanning the membrane once with a Nexo/Ccyt topology [von Heijne and Gavel (1988) Eur. J. Biochem. **174**, 671–678]. Overexpression of either wild-type or tagged Scamper induces a specific phenotype characterized by the rapid extension of actin-containing protrusions, followed by cell death.

Key words: cell protrusions, Niemann–Pick disease, sphingoid molecules.

INTRODUCTION

In the last decade, crucial events in cell life, such as proliferation, differentiation and apoptosis, have been shown to be controlled, among other agents, by various sphingoid molecules working as either first or second messengers [1–3]. The search for the corresponding specific receptors has led to the discovery of a number of proteins. Sphingosine 1-phosphate, produced by an intracellular sphingosine kinase [4], was recognized to act as an extracellular messenger binding to various G-protein-coupled receptors of the edg family [5,6]. Depending on the type of receptor and the nature of the coupled heterotrimeric G-protein, activation of either adenylate cyclase or phospholipase C pathways was shown to take place [7]. In contrast with sphingosine 1-phosphate [8], little is known about sphingosylphosphocholine (SPC), also referred to as 'sphingosylphosphorylcholine' or 'lysosphingomyelin'. SPC has been known for a long time to share with sphingosine 1-phosphate the ability to bind (although with lower affinity) and activate edg receptors [9]. The recent discovery of a G-protein-coupled receptor specific for SPC [10] has, however, suggested that SPC possesses properties independent of sphingosine 1-phosphate. With the exception of cardiac muscle, where it was proposed to exert a regulatory role [11], SPC is barely detectable in normal tissues [12]. Interestingly, in the neuropathic Niemann–Pick type A disease, SPC accumulates in liver, spleen and, most remarkably, in brain, where it appears to exert a toxic effect on neurons, leading to the appearance of severe neurological symptoms [13]. Since sphingomyelinase deficiency is the molecular hallmark of this disease [14,15], SPC accumulation has been proposed to arise from non-enzymic degradation of sphingomyelin [13].

The action of the two sphingoids within the cell is of particular interest. Their Ca^{2+}-release effect can be induced, in fact, not only via Ins(1,4,5)P_3 generated at surface receptors, but also by the action of the messengers themselves on intracellular Ca^{2+} stores [16]. This mechanism appears of great interest also because a potent mitogenic activity has been shown to be induced after its activation [17–19]. Initially, the effect of SPC was thought to be due to its interaction with ryanodine receptors [11,20] but not Ins(1,4,5)P_3 receptors [16,19,21]. Recently, however, experiments carried out in *Xenopus* oocytes have shown that the SPC-induced Ca^{2+} release depends on the expression of a new protein called 'SCaMPER' [22]. Although the original name stood for '**s**phingolipid **Ca**$^{2+}$-release-**m**ediating protein of the **e**ndoplasmic **r**eticulum', no evidence for its intracellular localization was provided in the original paper [22], and we have chosen to use the name 'Scamper' in the present paper.

In the present study we cloned the Scamper open reading frame to clarify both the subcellular distribution and the physiological relevance of the protein. Here we report a comprehensive study of Scamper, beginning with its primary structure and membrane topology. The morphological phenotype observed

Abbreviations used: BHK, baby-hamster kidney; (E)GFP, (enhanced) green fluorescent protein; IRES, internal ribosome entry site; MDCK, Madin–Darby canine kidney; RSV-LTR, rous-sarcoma-virus long tandem repeat; RT-PCR, reverse-transcription PCR; SPC, sphingosylphosphocholine; in the present paper we have used 'Scamper' rather than the original term 'SCaMPER' because the latter is linked to a function (sphingolipid Ca^{2+}-release-mediating) that has yet to be confirmed and a subcellular localization (endoplasmic reticulum) that we have proved to be unlikely.

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in the cell upon the overexpression of Scamper is also discussed.

EXPERIMENTAL

Materials

Anti-(green fluorescent protein) (Anti-GFP) (7.1 and 13.1) and anti-HA1 epitope (12CA5) monoclonal antibodies were from Boehringer-Mannheim, Mannheim, Germany; anti-(transferrin receptor) monoclonal antibody (H64.4) was from Zymed Laboratories, San Francisco CA, U.S.A.; rhodamine-conjugated phalloidin was from Sigma, Milan, Italy; anti-(protein disulphide-isomerase) polyclonal antibody [23] was a gift from Dr R. Sitia, Dibit, San Raffaele Scientific Institute, Milan, Italy; anti-giantin monoclonal antibody (GI/133) was a gift from Dr H. P. Hauri, Department of Pharmacology and Neurobiology, Biozentrum, Basel, Switzerland. Secondary rhodamine-conjugated goat anti-mouse and goat anti-rabbit antibodies were from Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A. Secondary horseradish-peroxidase-conjugated antibodies were from Bio-Rad, Hercules, CA, U.S.A. LysoTracker® Blue DND-22 was from Molecular Probes Europe BV, Leiden, The Netherlands. PCR on cDNA was performed using AmpliTaq DNA polymerase from PerkinElmer, Norwalk, CT, U.S.A.; oligonucleotides were purchased from MWG Biotech, Ebersberg, Germany. The plasmid pBat-4 was a gift from Dr J. Peränen, Institute of Biotechnology, University of Helsinki, Helsinki, Finland [24]. The MVA-T7pol vaccinia virus was kindly provided by Dr G. Sutter, Institute of Molecular Virology, GSF-National Research Centre for Environment and Health, Oberschleissheim, Germany [25]. Superfect transfection reagent was from Qiagen, Milan, Italy. Enhanced GFP (EGFP)-containing plasmids were from ClonTech, Palo Alto, CA, U.S.A. Cell-culture media and supplements were from Gibco BRL, Gaithersburg, MD, U.S.A. Restriction and modification enzymes for DNA cloning were from New England Biolabs, Beverly, MA, U.S.A.

Cell culture

Baby-hamster kidney (BHK-21) cells were maintained in Glasgow minimal essential medium supplemented with 5% (v/v) foetal-calf serum, 10% tryptose/phosphate broth, sodium Hepes, pH 7.2. Madin–Darby canine kidney (MDCK-II) cells were grown in minimal essential medium with Earle's salts supplemented with 5% foetal-calf serum. All media were supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and glutamine (2 mM). Cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere.

cDNA cloning and sequence analysis

Cytoplasmic RNA was isolated from MDCK cells as described by Wilkinson [26] and reverse-transcribed into cDNA by priming with oligo(dT). The primers scamp-se (CCA GAA TTC ATC ATA TGT TAA AAG) and scamp-as (GTT ATC TGC AGA TTT TCA ACC ATG) were used to amplify the cDNA by PCR. The following oligonucleotides were synthesized on the basis of information from GenBank® accession number U33628 in order to amplify 5′ and 3′ ends of the cDNA: scamp-s6 TTA AGA TAC TTT TTC TAA AAA GAT TTA T; scamp-a1 AGA GAG GTA CCT TTT AAG AGA GGA A; scamp-sa ATG GTT GAA AAT CTG CAT ATA AC; scamp-a3 TTT TTT TTT TAA GAT TTT ATG TAT TTA TTC. The PCR products were cloned in either pGEM-1 (digested with *Eco*RI and *Bam*HI) or pGEM-T vectors from Promega, Madison, WI, U.S.A., giving respectively pGEM-Scamper and pScamper. DNA sequencing was performed on both strands with Sequenase or ThermoSequenase (Amersham Biosciences, Piscataway, OH, U.S.A.). Sequencing reactions were also carried out in parallel by BioStrands, Trieste, Italy.

Construction of tagged Scamper

A plasmid containing 'HA-Scamper' under the control of the rous-sarcoma-virus long tandem repeat (RSV-LTR) promoter was prepared from a triple ligation of the *Xba*I–*Sac*I fragment from pOPRSVI-1 [27], a *Sac*I–*Nde*I fragment from pOPR-HA-Bene (D. Zacchetti, unpublished work), and the fragment *Nde*I–*Spe*I from pScamper, giving pOP-HASca. To prepare HA-Scamper under the control of the T7 promoter, the Scamper open reading frame from pScamper (*Nde*I filled-*Sal*I) was cloned into the *Bam*HI filled-*Sal*I pRmHa-N vector [28] giving pRmHa-Nscamper. Then the His-HA-tagged Scamper was removed with *Nco*I–*Stu*I and cloned into pBat-4 (*Nco*I-*Bam*HI filled) giving pBat-NScamper. The His tag was then removed by opening pBat-NScamper with *Nhe*I–*Bgl*II, filling with T4 DNA polymerase and re-ligating to produce pBat-VSca. To tag Scamper at the N-terminus with EGFP, we first cloned the EGFP from the pEGFP-C3 (*Kpn*I–*Xho*I) into pOPRSVI-1 (*Xba*I–*Xma*I) giving pOPR-EGFPC3. Scamper was then removed from pOP-HASca (*Kpn*I–*Xho*I) and cloned into pOPR-EGFPC3 (*Kpn*I–*Xho*I), and, later, the frame between EGFP and Scamper was restored by cutting with *Nde*I, filling, and rejoining, giving pOPR-EGFPSca. EGFP-Scamper was then removed by *Nco*I and inserted into pBat-4 (*Nco*I) for expression with T7pol recombinant vaccinia virus.

For C-terminus tagging of Scamper, a modified pBat-4 vector was prepared by cutting with *Nco*I, removing the overhangs with mung-bean (*Phaseolus aureus*) nuclease, and closing again the plasmid, giving pBat-mod. The Scamper open reading frame was amplified by PCR on pScamper with T7 and Scamp-ax (ACT GGC CCC GGG AGA GAG GAA GCA CTG CTT CAT C) as forward and reverse primers respectively, and cloned into pYX012 from Novagen, Madison, WI, U.S.A. (*Eco*RI–*Xma*I) to introduce an HA1 tag. Then the Scamper-HA was removed (*Eco*RI–*Xho*I) and cloned into the pBat-mod, giving pBatmod-ScaHA. pBatmod-ScaHA was then digested (*Sma*I–*Xho*I), and EGFP, extracted (*Age*I filled-*Bgl*II) from pEGFP-C3, fused in-frame. A convenient stop codon was introduced at the same time by using two synthetic oligonucleotides (BglpolyXho-s/poly-as GAT CTC TAG CTA GCC CGG GAA T/TCG AAT TCC CGG GCT AGC TAG A) giving pBatmod-ScaLEGFP. To remove the linker between Scamper and the EGFP, pBatmod-ScaLEGFP was digested (*Cla*I–*Nco*I) and a PCR fragment obtained from the same plasmid (sScashort/ScaGnoLas GGA GAT ATA TCG ATA TCG AAT TCA T/ACT GAC CAT GGA GAG GAA GCA CTG CTT CAT CTC A) was inserted to obtain pBatmod-ScamperEGFP.

Finally, a bicistronic construct was prepared by digesting pBatmod (*Xho*I–*Not*I) and inserting an internal ribosome entry site (IRES) obtained by *Xho*I–*Not*I digestion of pIRES2-EGFP, giving pBatmod-IRESEGFP. The Scamper open reading frame from pScamper (EcoRI-BglII) was then inserted in pBatmod-IRESEGFP (*Eco*RI–*Bam*HI), giving pBatmodScaIRESEGFP.

Overexpression of proteins

Cells were plated the day before an experiment in order to reach about 70% confluence at the time of transfection. Cells were

washed with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.9 mM KH_2PO_4) supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and infected with the MVA-T7pol virus in minimal essential medium for 30 min at 37 °C. After washes with PBS, cells were transfected with plasmids carrying the DNA construct of interest under the T7 promoter. The transfection was performed with Superfect according to manufacturer's instructions. The same protocol, but omitting the infection step, was used for expression of genes under the RSV-LTR promoter.

Immunofluorescence microscopy

Cells grown on coverslips were washed with PBS, fixed in 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After an additional wash with PBS, cells were blocked in 0.2% gelatin in PBS for 15 min and then incubated for 60 min with either a primary antibody (2.5 μg/ml) or rhodamine-conjugated phalloidin (2 μg/ml). After extensive washing (3×10 min), primary antibodies were visualized with goat anti-mouse or goat anti-rabbit rhodamine-conjugated antibodies (5 μg/ml, 45 min incubation, followed by extensive washing before mounting). Permeabilization prior to fixation was performed as described by Bucci and colleagues [29], with minor modifications. Briefly, cells plated on 24 mm-diameter round coverslips were washed once with PBS, permeabilized with 0.1% saponin in 80 mM potassium Pipes, pH 6.8, 5 mM sodium EGTA and 1 mM $MgCl_2$ for 5 min at 4 °C, and then fixed in 4% paraformaldehyde in PBS, pH 7.4, for 15 min. Blocking and antibody incubations were performed as described above. For vital staining, cells were washed with PBS and then incubated with 1 mM LysoTracker® Blue DND-22 in minimal essential medium with 0.1% BSA for 45–60 min at 37 °C. Fluorescence images were taken with a $63\times$-magnification, 1.4-numerical-aperture objective lens on an Axiovert 135 inverted microscope (Carl Zeiss, Cologne, Germany) equipped with an Orca-II digital camera (Hamamatsu Photonics, Hamamatsu-City, Japan).

SDS/PAGE and Western-blot analysis

Cells were detached with trypsin and disrupted in cold homogenization buffer (0.25 M sucrose/3 mM imidazole, pH 7.4) supplemented with a cocktail of protease inhibitors (pepstatin A, antipain, chymostatin and leupeptin, each at 10 μg/ml) by passing them several times through an 18-gauge needle. The cell homogenate was first centrifuged for 5 min at 2000 ***g*** to eliminate the nuclei. The supernatant was then ultracentrifuged for 1 h at 150000 ***g*** to separate the cytosolic fraction from the particulate. After 5 min denaturation at 95 °C in loading buffer (50 mM Tris/HCl, pH 6.8, 2% SDS, 5% glycerol, 2.5 mM sodium EDTA, 100 mM dithiothreitol and 0.5 mg/ml Bromophenol Blue), samples were subjected to SDS/PAGE and proteins transferred on to nitrocellulose membrane (Protran BA 85, 0.45 μm pore size; Schleicher and Schuell, Dassel, Germany) for 60 min on ice with a Genie electrophoretic blotting chamber (Idea Scientific Company, Minneapolis, MN, U.S.A.). After Ponceau Red staining, the membrane was blocked with 5% skimmed milk in washing buffer (10 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20), probed with the appropriate primary antibodies, and visualized with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL®) detection kit [Amersham Pharmacia Biotech (now Amersham Biosciences), Little Chalfont, Bucks., U.K.]. Incubation times and antibody dilutions were those recommended by the manufacturer.

RESULTS

Cloning and analysis of Scamper cDNA

The open reading frame of the Scamper cDNA was initially cloned by reverse-transcription PCR (RT-PCR) according to the published sequence [22], starting from the mRNA of MDCK cells. Since our sequence appeared distinct from that available in the database (GenBank® accession number U33628), we decided to clone also the 5′ and 3′ untranslated regions of the transcript to verify its origin from the same transcriptional unit. Initially, cloning of full-length cDNA was hindered by several problems. In general, the relative low abundance of the transcript in MDCK cells (Northern-blot analysis; results not shown) required the employment of several RT-PCR cycles with various primers and re-amplification steps, as described in detail in the Experimental section. In addition, standard sequencing reactions at the 5′ untranslated region, as well as primer extension with the T7 DNA polymerase, were unsuccessful, possibly because of the peculiar RNA secondary structure. The hypothesis that a strong secondary structure associated with high free energy was present in the part of the mRNA close to the 5′ end was also supported by specific predictions by the Mfold server [30]. Indeed this seemed to be the case, as sequencing problems were overcome when the reactions were carried out at higher temperature with *Taq* polymerase.

Further problems were encountered when sequencing the 3′ untranslated region of the Scamper mRNA, particularly after the stretch of adenine (resembling polyadenylation) at bases 1270–1293 of the U33628 database entry [22]. The possibility that the 3′ end of the transcript is shorter than previously reported was further strengthened by the presence of a consensus signal for polyadenylation at the right distance from the poly(A) stretch (bases 1261–1266 of the U33628 database entry and bases 1221–1226 of the AF263546 entry). The cloned sequence is described in Figure 1, and all inconsistencies with the findings of Mao and colleagues [22] are summarized under the Genbank accession number AF263546. Of particular relevance is the lack of a base in position 710, since it induces a frameshift and generates a new stop codon. According to our sequence, a short primary structure, consisting of 110 amino acids, can be predicted for the Scamper protein (see details in Figure 1). On the basis of the hydropathy plot and the secondary-structure prediction by PHDsec [31] made available via the GeneQuiz server [32], only one α-helical transmembrane domain appears to be included in the protein. Since no clear signal sequence is present, the predicted transmembrane domain is expected to work as the signal anchor. Topology prediction with TMpred server [33] suggests an 'N-terminus-outside' model.

Topology and localization of the Scamper protein

On the basis of the new information obtained, experiments on Scamper were planned that were aimed at defining its topology and intracellular localization. Our initial strategy was to raise in rabbits anti-peptide antibodies against regions located on either side of the membrane. The first antibody, corresponding to the predicted amino acid sequence SITEAPDLKIRDPK, was affinity-purified and gave a strong signal in immunoblotting against the Scamper protein produced in *Escherichia coli* (results not shown). Unfortunately, the antibody was unable to recognize in eukaryotic cells either the endogenous or the overexpressed Scamper protein, possibly because of post-translational modifications. A second antibody against a peptide on the opposite side of the membrane (RNIFIYINRVRNIKR) failed to recognize even the Scamper protein produced in *E. coli*. To overcome the

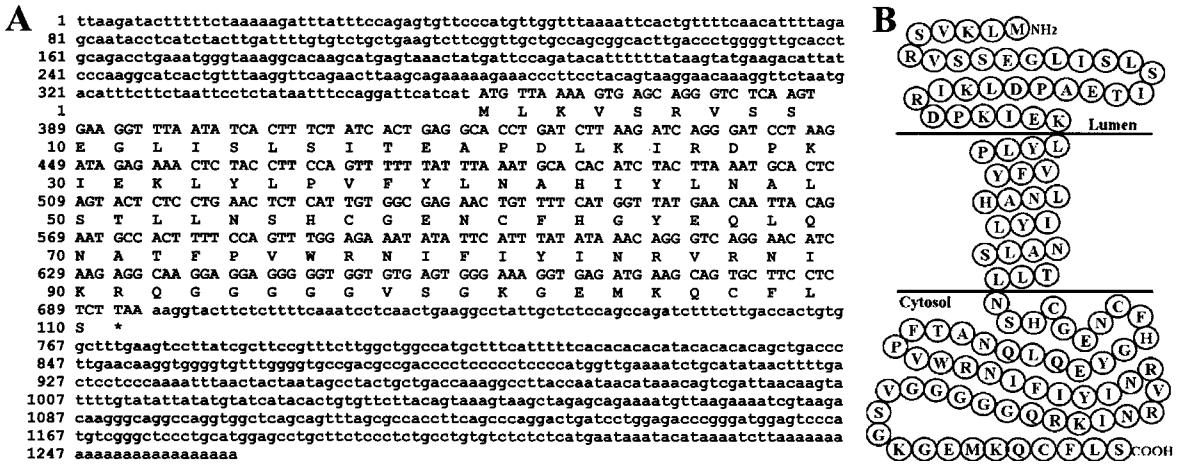

Figure 1 Molecular characterisation of Scamper cDNA

(**A**) Sequence of Scamper cDNA and deduced primary structure. The asterisk corresponds to the stop codon. (**B**) Corresponding 'snake' diagram based on the hydropathy plot and the TMpred output.

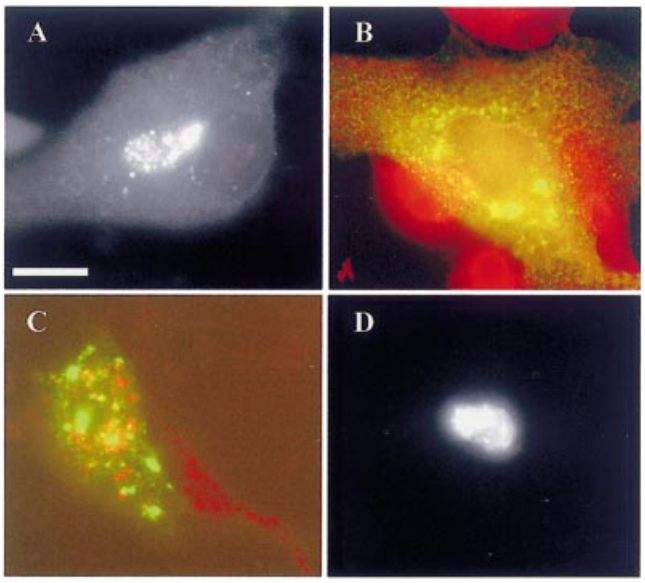

Figure 2 Fluorescence localization of Scamper tagged at the N-terminus with EGFP (EGFP-Scamper) and overexpressed in BHK-21 cells

(**A**) Fluorescence localization of EGFP-Scamper. (**B**, **C**) lack of co-localization of EGFP-Scamper (in green) with the endoplasmic-reticulum marker protein disulphide-isomerase (red in **B**) or the marker for acidic compartments LysoTracker Blue® (red in **C**). (**D**) Example of rounding and shrinkage of a cell following longer EGFP-Scamper overexpression. The bar represents 10 μm in all panels.

problem, we transiently overexpressed the Scamper protein fused with either the HA1 epitope tag from influenza haemagglutinin [34] or EGFP [35]. Two different transfection protocols (RSV-LTR promoter and MVA-T7pol vaccinia virus expression system) were used. With Scamper tagged at the N-terminus with EGFP (EGFP-Scamper) the initial staining of BHK cells was in intracellularly dispersed small dots that progressively coalesced into bigger intracellular structures localized in the perinuclear region (Figure 2A). Double-staining experiments, performed at early time points of expression, failed to reveal any co-localization with markers of the endoplasmic reticulum [anti-(protein disulphide-isomerase) polyclonal antibody] and of the acidic endosomal/lysosomal compartment (Lysotracker® Blue) (Figures 2B and 2C). Longer expression times (more than 8 h with MVA-T7pol and more than 18 h with RSV-LTR) led to rounding and shrinking of the transfected cells (Figure 2D). The kinetics of the expression, the localization dynamics, as well as the general cell phenotype, suggested the formation of aggresomes, i.e. aggregates formed by overexpression of misfolded cytosolic proteins [36,37]. The same results were obtained when Scamper was tagged at the N terminus with the HA1 tag (HA-Scamper; results not shown).

Tagging at the C terminus (Scamper-HA and Scamper-EGFP) gave different results. While the HA tag could not be detected by conventional immunostaining, the EGFP signal was evenly distributed within the cell, suggesting that the fusion protein might have released the fluorophore from the membrane (Figure 3A). This hypothesis was confirmed by Western-blotting detection of the EGFP (Figure 3B), which showed that the majority ($> 60\,\%$) of the fusion protein had undergone proteolytic cleavage, with recovery of the EGFP moiety in the soluble cytosolic fraction. To localize the uncleaved Scamper–EGFP protein, we used a permeabilization protocol modified from Bucci and colleagues [29] that allows free cytosolic components to leave the cell. By this procedure, membrane proteins are not solubilized. Cytosolic EGFP and an integral membrane protein, VIP17/MAL [38], fused with EGFP, were used as positive and negative controls respectively to set up the procedure (results not shown). This permeabilization protocol was effective in unmasking the full-length Scamper–EGFP fusion protein (Figure 3C), but significantly affected the morphology of membrane-bounded organelles, causing extensive intracellular vesiculation. Double-staining experiments confirmed that the intracellular localization of Scamper does not coincide with the endoplasmic reticulum (Figure 3D). Also, no clear co-localization with markers of the endosomal pathway (Figure 3E) or the Golgi complex (Figure 3F) was observed.

Noticeably, overexpression of Scamper neither affected the basal intracellular Ca^{2+} homoeostasis nor induced sensitivity to the exogenous administration of SPC in MDCK cells (results not shown).

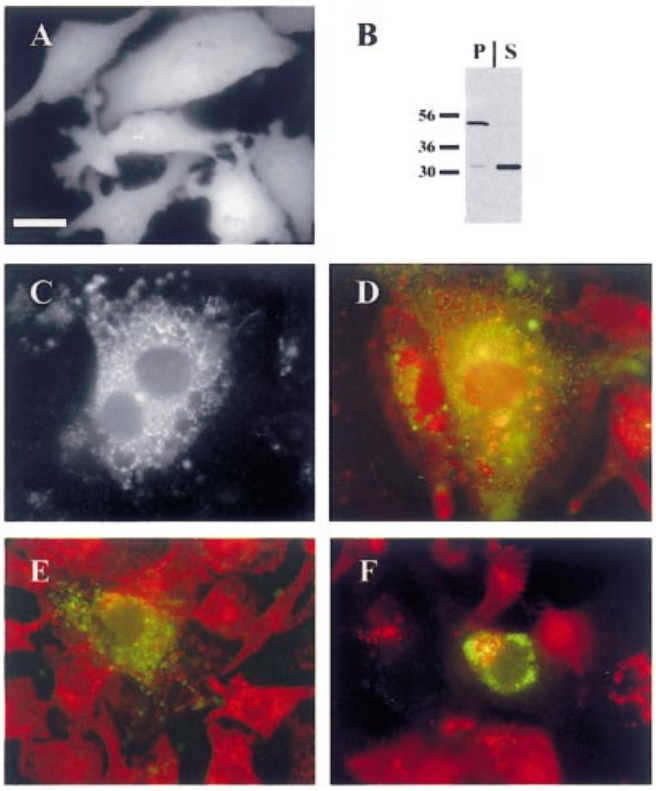

Figure 3 Overexpression of Scamper tagged at the C-terminus with EGFP (Scamper-EGFP) in BHK-21 cells

(**A**) Fluorescence signal from cells overexpressing Scamper-EGFP. (**B**) Western-blot analysis of cells overexpressing Scamper-EGFP. The homogenates of transfected BHK-21 cells were fractionated by ultracentrifugation and proteins probed by anti-EGFP antibodies after SDS/15%-(w/v)-PAGE separation and transfer on to nitrocellulose membrane. The pellet (lane P) contains the membrane fraction and the supernatant (lane S) the cytosol. The positions of molecular-mass markers (in kDa) are indicated at the left. (**C**) Controlled permeabilization of BHK-21 cells with saponin (0.1%) releases cytosolic EGFP and reveals intracellular location of Scamper-EGFP. (**D**–**F**) Immunofluorescence of transiently transfected BHK-21 cells permeabilized before fixation. Green staining is due to the membrane-bound Scamper-EGFP, while red staining is due to the specific marker for: the endoplasmic reticulum (protein disulphide-isomerase; **D**); the endosomal pathway (transferrin receptor; **E**); and the Golgi complex (giantin; **F**). The bar represents 10 μm in all the panels.

Morphological alterations and toxicity induced by Scamper overexpression

Expression of Scamper-EGFP with MVA-T7pol for more than 6 h caused the progressive appearance of BHK-cell phenotype alterations. In particular, the presence of thin cell protrusions was also observed by epifluorescence, thanks to the strong cytosolic EGFP staining due to cleavage of the fusion protein (Figure 4A). Interestingly, this phenotype was also reproduced by the expression of wild-type Scamper and EGFP using a bicistronic construct (Figure 4B). Similar expression experiments were performed in another system, i.e. MDCK cells, from which Scamper cDNA had been cloned (Figure 5). The protrusions of transfected cells were characterized by their actin content (Figure 5B).

Finally, times of overexpression of Scamper in BHK cells (alone or coupled to EGFP) longer than 12 h led to more severe alterations in morphology, followed by cell death (Figure 6). Similar results were obtained in other cell lines, including MDCK cells (results not shown). In contrast, EGFP alone failed to induce any cell toxicity under the same experimental conditions (results not shown). Interestingly, no toxicity was observed when Scamper was overexpressed in *E.* coli (results not shown).

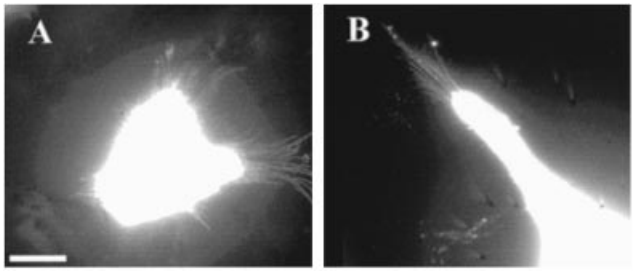

Figure 4 Morphological alterations induced by Scamper overexpression

(**A**) Fluorescence image of a BHK-21 cell overexpressing Scamper-EGFP. (**B**) Fluorescence image of a BHK-21 cell overexpressing the wild-type Scamper. The bar represents 10 μm in both panels.

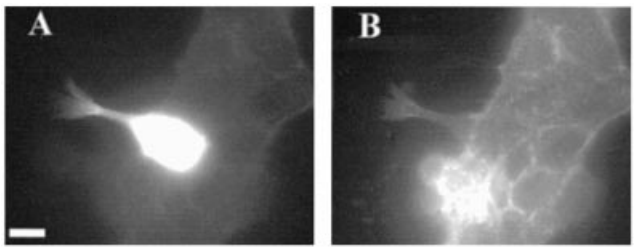

Figure 5 Scamper-EGFP overexpression in MDCK cells

Fluorescence images of Scamper-EGFP overexpression in MDCK cells with concomitant actin staining. (**A**) EGFP signal; (**B**) rhodamine–phalloidin staining. The bar represents 10 μm in both panels.

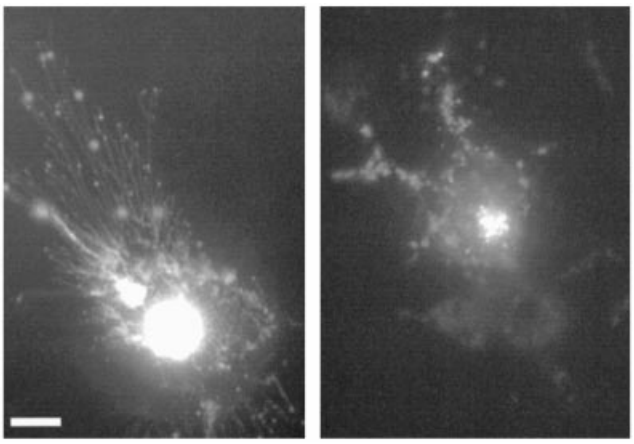

Figure 6 Toxicity induced by Scamper overexpression

Examples of severe alteration in cell morphology leading to cell death after more than 12 h of Scamper-EGFP overexpression in BHK cells using the MVA-T7pol expression system. The bar represents 10 μm in both panels.

DISCUSSION

In the present work we have re-examined the properties of Scamper, a proposed new member of the intracellular Ca^{2+} channel family. Our interest in this molecule stems from the physiological importance of signalling pathways controlling Ca^{2+} release from intracellular stores. Scamper was expected to be of particular relevance because it had been proposed as the final effector of the intracellular SPC signalling pathway, a route that, although involved in important cell activities, still awaits clear definition. Surprisingly, after the initial characterization by Mao and colleagues [22], no additional work was published on Scamper, even though its role as intracellular Ca^{2+} channel was widely accepted and recognized in the most recent reviews [39,40].

In our studies Scamper was rapidly recognized to be quite different from its initial description. Discrepancies between our sequence and the one previously published were so fundamental that a re-examination of the gene product, regardless of the putative function as intracellular SPC receptor, was imperative to create a basis for Scamper characterization. In fact, our predicted primary structure was significantly shorter than the published one. Moreover, the secondary structure exhibited one (and not two) α-helical transmembrane domains – an organization never been reported for an ion channel. Here we do not intend to report in detail about the multiple discrepancies between the published Scamper sequence and ours. They most likely took origin from the different experimental strategies employed in the two studies. It is worth emphasizing that we used a direct RT-PCR approach, while Mao and colleagues [22] cloned the Scamper cDNA from a library, an approach that is intrinsically more prone to artifacts. Moreover, several independent amplification experiments produced identical sequences, thereby ruling out the prospect that amplification artifacts might have affected our results. Recently, the sequencing of the clone used by Mao and colleagues [22], performed after the disclosure of our sequence in the Genbank database (accession number AF263546), confirmed the presence of sequencing mistakes in the original entry submitted with the accession number U33628 (R. Betto, personal communication).

Further discrepancies arose when we investigated topology and intracellular localization of Scamper. These experiments were problematic, because of the lack of suitable antibodies. In addition, the epitope-tagging strategy did not give straightforward results. Tagging of the Scamper N-terminal with EGFP or HA1 did not provide useful information, since it led to staining of structures that strongly resemble aggresomes, the recently discovered intracellular compartments that form *de novo* on accumulation of misfolded proteins [36,41]. On the other hand, immunolocalization of the C-terminal tagged Scamper was complicated by proteolytic processing of the fusion protein, with release of the tag (EGFP) into the cytosol. This serendipity provided direct confirmation of a type III (Nexo/Ccyt) topology [42], in contrast with the type II (Ncyt/Cexo) proposed by Mao and colleagues [22]. Unfortunately, the cytoplasmic EGFP masked the intracellular membrane localization, which was revealed only after controlled permeabilization with saponin and release of the cytosolic signal. Although a complete set of experiments with markers of the various intracellular compartments has not been performed, our data clearly indicate that the Scamper leaves the endoplasmic reticulum after synthesis. The possibility that EGFP tagging at the C-terminus might have led to mistargeting of the protein is highly unlikely, since the overexpression of unmodified Scamper induces the same phenotype characterized by the presence of actin-containing protrusions. This result is intriguing, considering that a Prosite search [43] predicts an actinin-type actin-binding domain in the Scamper primary structure. However, it is impossible to speculate at this stage whether this phenotype represents a 'gain of normal function' or simply an unrelated effect that follows Scamper overexpression.

In any event, soon after the massive production of the actin-containing protrusions the cells die, thus showing that overexpression of either wild-type Scamper or Scamper tagged at the C-terminus is toxic. This is no surprise, since the problems encountered in unambiguously detecting bands in Northern blots, as well as the scarcity of homologous cDNAs in expressed-sequence-tag ('EST') databases, indicate low levels of expression of the transcript. In line with these considerations, we have evidence that the unusually long 5′ untranslated region of Scamper mRNA might exert a tight control on the protein expression at the translational level (D. De Pietri Tonelli and D. Zacchetti, unpublished work). Also, the proteolytic cleavage of the Scamper protein that occurs close to the C-terminus could represent an important physiological post-translational regulation of Scamper function. It is not known whether this cleavage is related to cell death and activation of the caspase cascade or to some other proteolytic process [44]. In any event this issue deserves further investigation.

The evidence presented clearly makes the proposal of Scamper as a new member of the intracellular Ca^{2+} channel family most unlikely. First of all, the secondary structure, with only one predicted transmembrane domain, is hardly compatible with our present knowledge of the molecular organization of ion channels [45]. Secondly, it is clear that the protein does not reside within the endoplasmic reticulum, the organelle generally thought to be responsible for intracellular Ca^{2+} storage and release [46]. There remains the possibility that Scamper might participate in the modulatory role which SPC is claimed to exert on ryanodine receptors [20], even though, at present, there is no evidence.

In conclusion, our work sheds some light on what Scamper is not, and, looking to the future, raises the possibility of discovering the function of this protein, which, although at present still elusive, promises to be of great significance for cell homoeostasis.

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