Analysis of SCAMP1 Function in Secretory Vesicle Exocytosis by Means of Gene Targeting in Mice*

(Received for publication, April 28, 1999, and in revised form, July 22, 1999)

Rafael Fernández-Chacon‡‡, Guillermo Alvarez de Toledo‡, Robert E. Hammer‡, and Thomas C. Südhof‡‡‡

From the §Center for Basic Neuroscience, Department of Molecular Genetics, ¤Department of Biochemistry, and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, Texas 75235 and the Departmento de Fisiología Medica y Biofísica, Facultad de Medicina, Universidad de Sevilla, Avda. Sanchez-Pizjuan 4, 41009 Sevilla, Spain

Secretory carrier membrane proteins (SCAMPs) comprise a family of ubiquitous membrane proteins of transport vesicles with no known function. Their universal presence in all cells suggests a fundamental role in membrane traffic. SCAMPs are particularly highly expressed in organelles that undergo regulated exocytosis, such as synaptic vesicles and mast cell granules. Of the three currently known SCAMPs, SCAMP1 is the most abundant. To investigate the possible functions of SCAMP1, we generated mice that lack SCAMP1. SCAMP1-deficient mice are viable and fertile. They exhibit no changes in the overall architecture or the protein composition of the brain or alterations in peripheral organs. Capacitance measurements in mast cells demonstrated that exocytosis could be triggered reliably by GTPγS in SCAMP1-deficient cells. The initial overall capacitance of mast cells was similar between wild type and mutant mice, but the final cell capacitance after completion of exocytosis, was significantly smaller in SCAMP1-deficient cells than in wild type cells. Furthermore, there was an increased proportion of reversible fusion events, which may have caused the decrease in the overall capacitance change observed after exocytosis. Our data show that SCAMP1 is not essential for exocytosis, as such, and does not determine the stability or size of secretory vesicles, but is required for the full execution of stable exocytosis in mast cells. This phenotype could be the result of a function of SCAMP1 in the formation of stable fusion pores during exocytosis or of a role of SCAMP1 in the regulation of endocytosis after formation of fusion pores.

SCAMPs\(^1\) represent a family of membrane proteins present in transport vesicles (1–7). The three SCAMPs that have been identified in mammals (2) are proteins of 35–40 kDa that are composed of an N-terminal cytoplasmic sequence followed by four transmembrane regions and a short cytoplasmic tail. SCAMPs are highly conserved evolutionarily and very homologous to each other. Although they were originally discovered as components of secretory granules in exocrine glands (8), SCAMPs were later found to be present in all cells tested on post-Golgi transport vesicles (3–7). SCAMPs are particularly abundant in vesicles that are subject to regulated exocytosis, such as mast cell granules, exocrine granules, and synaptic vesicles. The three SCAMP proteins are differentially expressed in tissues and are not universally co-expressed in all cells (2). Only one of the three SCAMPs, SCAMP1, appears to be present at high levels on synaptic vesicles that lack SCAMPs 2 and 3 (Refs. 1 and 2 and footnote 2).

The function of SCAMPs are unknown. Their ubiquitous presence in transport vesicles in all cells indicates a fundamental role in membrane traffic. The enrichment of SCAMPs in secretory vesicles that are subject to regulated exocytosis followed by rapid endocytosis suggests a function in exo- or endocytosis. Interestingly, SCAMP1 and SCAMP3 are tyrosine-phosphorylated \textit{in vivo} by the epidermal growth factor receptor tyrosine kinase (9); this result indicates that SCAMPs may be targets of intracellular signal transduction pathways. To gain insight into the functions of SCAMPs, we have used homologous recombination to abolish SCAMP1 expression in mice. Surprisingly, SCAMP1-deficient mice were viable and fertile. The knockout mice exhibited no major nervous system phenotypes despite the fact that SCAMP1 is the major, and possibly only, SCAMP isoform on synaptic vesicles. Furthermore, capacitance recordings in mast cells that normally express copious amounts of SCAMP1 (10) revealed that SCAMP1 is not essential for the generation or maintenance of normally sized secretory vesicles nor is it required for exo- or endocytosis as such. The only phenotype observed was a change in the total amount of stable membrane fusion that could be triggered by GTPγS and in the frequency of reversible fusion events in SCAMP1-deficient mast cells during exocytosis.

**MATERIALS AND METHODS**

\textit{Molecular Cloning of the Marine SCAMP1 Gene, Construction of a Knockout Vector, and Generation of SCAMP1 Knockout Mice—A mouse genomic library (λ-FIX, Stratagene) was screened with a uniformly \textit{32P}}-labeled probe from the rat SCAMP1 cDNA (1, 11, 12). Mapping and sequencing revealed that one genomic clone (pmlSCAMP1–4) subcloned into pBluescript contained exons 3–5 from the N-terminal coding region of SCAMP1. Exon numbers were identified by comparison with the published pig SCAMP1 gene (see Fig. 1A and Ref. 13). A targeting vector was designed to delete exons 3 and 4 (residues 46–114 of SCAMP1). The vector contained neomycin resistance and diphtheria toxin gene cassettes as positive and negative selectable markers, respectively (Fig. 1A). ES cells (SM1 cells from Sv/129 mice) were cultured on irradiated STO cells and electroporated with NotI-linearized targeting vector as described (12, 14, 15). After selection with 0.19 g/liter G418, resistant clones were picked, expanded, and analyzed by Southern blotting of genomic DNA digested with EcoRI, BamHI, or XbaI with an 0.57-kb \textit{NotI–PstI} fragment from the 5′-end of pmlSCAMP1–4 as an outside probe to detect homologous recombination (Fig. 1). Polymerase

\(^1\) The abbreviations used are: SCAMP, secretory carrier membrane protein; ES cells, embryonic stem cells; kb, kilobase (pair); F, farad (F, \(\mu F, pF\)).
chain reaction genotyping was performed in a single tube with three oligonucleotides. Sequences were: 1544 = GTCTCTGTCTCTCTCTCTTTCCTCA; 1545 = CTGCCAGGCCCTAGTCTCTACCG; 614 = GAGCGGGCGCGAGTGGTTAGG; 1544 versus 1545 = 0.70-kb wild type product; 1544 versus 614 = 0.44-kb mutant product. Chimeric mice derived from one ES cell clone transmitted the mutant gene through the germline; resulting offspring were used for all further studies. Mice were maintained using standard mouse husbandry (12, 14, 15).

Antibodies, Immunoblotting, and Immunocytochemistry—A GST-fusion protein containing the entire N-terminal cytoplasmic sequence of SCAMP1 (encoded by pGEK-SCAMP1) was used to generate a SCAMP1-specific antibody (R806) that did not react with SCAMP2 and SCAMP3 and was expressed in transfected COS cells (data not shown). Immunoblotting of total brain homogenates from wild type and mutant mice was carried out as described (16); signals were detected by 125I-labeled secondary antibodies. Immunocytochemical analysis was performed on cryostat brain sections from perfusion-fixed adult mice and developed using the peroxidase-antiperoxidase technique and heavy metal enhancement (12).

Morphological and Electrophysiological Analysis of Mast Cells—Mast cells were obtained essentially as described (17, 18) from wild type and SCAMP1 knockout mice by peritoneal lavage with buffer A containing (in millimolar; pH 7.2): 140 NaCl, 10 HEPE- NaOH, 2 CaCl2, 1 MgCl2, 6 glucose, 45 NaHCO3, and 0.4 sodium phosphate. For immunofluorescence analysis, mast cells were fixed in 4% paraformaldehyde, 0.1 M phosphate buffer, and 0.12 M sucrose for 20 min and permeabi-

FIG. 1. Generation of SCAMP1 knockout mice by homologous recombination. A, structures of the wild-type SCAMP1 gene, the targeting vector (Neo, neomycin resistance gene; DT, diphtheria toxin gene), and the mutant SCAMP1 gene after homologous recombination. Filled boxes labeled 3, 4, and 5 represent exons 3–5. The position of the outside probe for Southern blotting is indicated on the left. Arrows with numbers identify oligonucleotides used for polymerase chain reaction genotyping. Locations of selected restriction sites are shown; the scale is given on the lower right. B, Southern blot analysis of genomic DNA from ES cells with a wild-type SCAMP1 genotype (+/+), a heterozygous genotype (+/−), or a homozygous genotype (−/−). DNA was digested with EcoRI and BamHI and hybridized with the probe indicated in A. Numbers on the left indicate positions of molecular weight markers.

RESULTS AND DISCUSSION

Generation of SCAMP1 Knockout Mice—To construct a target-

We electroporated the targeting vector into ES cells and selected resistant clones with neomycin. Southern blotting analysis showed that 2.5% of the resistant clones were the result of homologous recombination (Fig. 1B). One of several clones injected into blastocysts gave rise to highly chimeric mice that transmitted the SCAMP1 mutation through the germline. The resulting heterozygous mice were bred to homozygous and analyzed by a number of techniques. Unexpectedly, we found that mice homozygous for the SCAMP1 mutation were viable and fertile. Matings between heterozygotes produced offspring with a normal Mendelian distribution of the mutant allele (data not shown). No increased morbidity was observed after prolonged periods of observation; even homozygous mutant mice 18 months of age exhibited no disease symptoms or increased mortality. These experiments demonstrate that SCAMP1 is not an essential gene.

The SCAMP1 Mutation Ablates Expression but Does Not Induce Changes in the Structure or Composition of the Brain—Considering the fact that SCAMP1 is the major SCAMP iso-

marker. The vector design predicts that homologous recombination should lead to a loss of the diphtheria toxin expression, a gain of neomycin resistance, and a deletion of exons 3 and 4. As a result, homologous recombination should remove residues 46–144 of SCAMP1. In addition, if exon 2 were spliced to exon 5 after homologous recombination, an out-of-frame junction in the mRNA would occur that should also abolish SCAMP1 expression.

We obtained a calibration signal by unbalancing the C-slow potentiometer. The final Ca2+ concentration was between 500 and 900 nM. We added 5–10 μM GTPγS to the pipette solution to induce degranula-

RESULTS AND DISCUSSION

Generation of SCAMP1 Knockout Mice—To construct a target-

We obtained a calibration signal by unbalancing the C-slow potentiometer. The final Ca2+ concentration was between 500 and 900 nM. We added 5–10 μM GTPγS to the pipette solution to induce degranula-

We electroporated the targeting vector into ES cells and selected resistant clones with neomycin. Southern blotting analysis showed that 2.5% of the resistant clones were the result of homologous recombination (Fig. 1B). One of several clones injected into blastocysts gave rise to highly chimeric mice that transmitted the SCAMP1 mutation through the germline. The resulting heterozygous mice were bred to homozygous and analyzed by a number of techniques. Unexpectedly, we found that mice homozygous for the SCAMP1 mutation were viable and fertile. Matings between heterozygotes produced offspring with a normal Mendelian distribution of the mutant allele (data not shown). No increased morbidity was observed after prolonged periods of observation; even homozygous mutant mice 18 months of age exhibited no disease symptoms or increased mortality. These experiments demonstrate that SCAMP1 is not an essential gene.

The SCAMP1 Mutation Ablates Expression but Does Not Induce Changes in the Structure or Composition of the Brain—Considering the fact that SCAMP1 is the major SCAMP iso-

We obtained a calibration signal by unbalancing the C-slow potentiometer. The final Ca2+ concentration was between 500 and 900 nM. We added 5–10 μM GTPγS to the pipette solution to induce degranula-

We obtained a calibration signal by unbalancing the C-slow potentiometer. The final Ca2+ concentration was between 500 and 900 nM. We added 5–10 μM GTPγS to the pipette solution to induce degranula-

We obtained a calibration signal by unbalancing the C-slow potentiometer. The final Ca2+ concentration was between 500 and 900 nM. We added 5–10 μM GTPγS to the pipette solution to induce degranula-

We obtained a calibration signal by unbalancing the C-slow potentiometer. The final Ca2+ concentration was between 500 and 900 nM. We added 5–10 μM GTPγS to the pipette solution to induce degranula-
Blots containing equivalent amounts of type mice and in mice heterozygous (\(+/\)) for the SCAMP1 mutation. Blots containing equivalent amounts of protein were analyzed with antibodies to the indicated proteins (\(\text{NMDA-R, N-methyl-D-aspartic acid receptor}\) and \(\text{125I-labeled secondary antibodies}\). Numbers on the left indicate the position of molecular weight markers. Note that the SCAMP monoclonal antibody reacts with a lower affinity with SCAMPs other than SCAMP1 (3); as a result, these other SCAMPs are apparent in the blot with the monoclonal antibody (arrowheads). \(\text{Sypl, synaptophysin I; Sypl, synaptotagmin I; Sypl, synaptobrevin II; Sypl, synapsin; Raph \text{3a}, rabphilin 3a; Cpx I, complexin I; Stx 1A, syntaxin 1A.}\)

Fig. 2. Immunoblotting analysis of synaptic proteins in wild-type mice and in mice heterozygous (\(+/-\)) or homozygous (\(-/-\)) for the SCAMP1 mutation. Numbers on the left indicate the position of molecular weight markers. Note that the SCAMP monoclonal antibody reacts with a lower affinity with SCAMPs other than SCAMP1 (3); as a result, these other SCAMPs are apparent in the blot with the monoclonal antibody (arrowheads). Sypl, synaptophysin I; Sypl, synaptotagmin I; Sypl, synaptobrevin II; Sypl, synapsin; Raph \text{3a}, rabphilin 3a; Cpx I, complexin I; Stx 1A, syntaxin 1A.

Essential Functions of SCAMP1

In general SCAMP monoclonal antibody and a SCAMP1-specific polyclonal antibody, we detected no SCAMP1 in the homozygous mutant and found reduced SCAMP1 protein levels in the heterozygotes (Fig. 2). Thus, the mutation we introduced is effectively a null mutation. The SCAMP monoclonal antibody reacts weakly with other SCAMP isoforms in addition to SCAMP1 (1–3). Analysis of the knockout mice shows, however, that these other SCAMP isoforms, which appear to be less abundant than SCAMP1, are unchanged in the knockout mice, suggesting that there is no compensatory change in these isoforms (arrowheads in the top panel in Fig. 2).

We then studied another series of synaptic proteins in the same samples. No major change in any of these proteins was recovered (Fig. 2 and data not shown). In particular, the levels of an array of synaptic vesicle proteins (synaptophysin, synaptotagmin, synaptobrevin, rabphilin, synapsins, Rab3α) exhibited no obvious alteration, nor were there changes in a cytosolic presynaptic protein (complexin) or postsynaptic \(\text{N-methyl-D-aspartic acid receptors}\). These results indicate that deleting SCAMP1 does not induce major aberrations in the brain. To confirm this conclusion by an independent method, we performed immunocytochemistry experiments with sections of the hippocampus from wild type and heterozygous SCAMP1 mutant mice. Sections were probed with antibodies to SCAMP1 and to synaptoporin (data not shown). When we compared the SCAMP1 staining patterns between wild type and SCAMP1 knockout mice, we found no SCAMP1 expression in the knockout mice, as expected. A comparison of the synaptoporin staining pattern, however, showed that the distribution of synaptoporin was indistinguishable between wild type and SCAMP1 knockout mice (data not shown). The synaptic vesicle protein synaptoporin is an isoform of synaptophysin that is highly concentrated in mossy fiber terminals of the CA3 region (21). It is therefore a marker that is sensitive to changes in the structural organization of the hippocampus. The absence of changes in the structure of the hippocampus or in the levels in major synaptic proteins indicates that the SCAMP1 knockout does not lead to a reorganization of the brain architecture.

Mast Cell Exo- and Endocytosis in SCAMP1 Knockout Mice—Mast cells are specialized secretory cells with large exocytotic granules and high levels of SCAMP1 (10). Mast cells have been very useful in electrophysiological studies in which membrane traffic is measured by capacitance changes (17, 18, 22–27). Immunocytochemical staining of peritoneal mast cells from wild type and SCAMP1 knockout mice with antibodies to SCAMP1 confirmed that SCAMP1 is enriched in the secretory granules of mast cells and is absent from the knockout animals (data not shown). Nevertheless, the SCAMP1-deficient mast cells had a normal appearance with no major changes in the distribution or sizes of secretory granules as judged by Nomarski optics.

To determine whether the SCAMP1 deficiency induced a functional defect in mast cell exo- and/or endocytosis, we performed capacitance measurements in SCAMP1-deficient mast cells by patch clamp electrophysiology (Fig. 3). As a control, we used mast cells from wild type littermates that have the same genetic background. Capacitance recordings allow monitoring of exo- and endocytosis in real time. In addition, the size of the granules and the kinetics of fusion can be determined by capacitance measurements in mast cells because of the large size of their secretory granules. Using capacitance recordings, we found that GTP\(\gamma\)S triggered exocytosis in SCAMP1-deficient mast cells as readily as in wild type mast cells (Fig. 3). In cells of both genotypes, capacitance changes occurred in discrete steps, which are thought to correspond in size to a single secretory granule (23–26). The average size of the individual capacitance steps was slightly but not significantly smaller in the knockout cells than in wild type cells. Accordingly, the
radius of the secretory granules calculated from these measurements was similar between wild type and mutant cells (see Fig. 3, C–F). These data show that mast cells lacking SCAMP1 contain normally sized secretory granules that are fusion competent.

We next quantified the overall capacitance changes that could be triggered by GTP•S in wild type and knockout mast cells. Cells with or without SCAMP1 exhibited a similar overall initial capacitance (Fig. 4A). Interestingly, however, the final capacitance was significantly different between wild type and knockout cells (Fig. 4B). Inspection of the capacitance traces indicated that there may be an increase in the frequency of reversible exocytosis in the knockout mice (asterisks in Fig. 3A). These reversible fusion events probably reflect transient fusion reactions (17) or accelerated membrane retrieval processes after exocytosis. Quantitation of the frequency of such events confirmed that the SCAMP1-deficient cells had a tendency to undergo more frequent fusion reversals, indicating that this may have contributed to the decrease in the overall capacitance change triggered by GTP•S (Fig. 4C).

**SUMMARY**

SCAMP1 is the most abundant SCAMP isoform in mammals and the only currently described isoform that is present in synaptic vesicles. In the present study, we generated a null mutant for SCAMP1 and analyzed its phenotype. Probably our most remarkable observation is that mice lacking SCAMP1 exhibit relatively few changes: SCAMP1 is clearly not an essential gene, is not required for the stability or normal size of secretory vesicles, and is not needed for exo- or endocytosis as such. In view of its abundance and universal expression, the relatively mild phenotype of the SCAMP1 knockout mouse is surprising. At least three hypotheses can be advanced to explain this finding. 1) SCAMP1 may be functionally redundant. Although the other two currently known SCAMP isoforms (SCAMP2 and SCAMP3) are not present in synaptic vesicles, additional SCAMPs may be expressed here and elsewhere that could be functionally redundant. 2) SCAMP1 may have a discrete specialized function that only becomes apparent in unusual circumstances. For example, it could be essential for particular environmental stresses that we have not tested. 3) SCAMP1 may have a very subtle, relatively unimportant function. Although rarely considered, evolutionary leftovers in the genome with relatively minor functions are conceivable. For example, even an abundant and conserved protein such as serum albumin can be deleted in humans without untoward consequences. Future studies will have to address which of these hypotheses is the most likely. Although SCAMP1 was found to be nonessential in our studies, its deletion did have phenotypic consequence for membrane traffic; we observed an increase in the frequency of reversible fusion events in mast cells and a decrease in the total capacitance change generated after stimulation of exocytosis, even though the starting capacitance was identical. This phenotype is distantly related to the phenotype observed in ruby mutant mice (27). It could be the result of a role for SCAMP1 in stabilizing fusion pores, a role that would agree well with the conserved transmembrane structure of SCAMPs (3, 13). Alternatively, this phenotype could be caused by a function of SCAMP1 in the timing of endocytosis, resulting in an increase of fast endocytosis when SCAMP1 is absent. Whichever of these models for the function of SCAMP1 is correct, either invokes a role for SCAMP1 in membrane traffic at the cell surface, which agrees well with its universal presence in small transport vesicles in all cells (1–3).

**Acknowledgments**—We thank Drs. J. D. Castle and R. Jahn for generous gifts of monoclonal antibodies, Drs. T. W. Rosahl, M. Missler, and R. Jahn for advice, and A. Roth, I. Leznicki, E. Borowicz, B. Perkins, and J. L. Romero for excellent technical assistance.

**REFERENCES**

1. Brand, S. H., Laurie, S. M., Mixon, M. B., and Castle, J. D. (1991) *J. Biol. Chem.* 266, 18949–18957
2. Brand, S. H., and Castle, J. D. (1993) *EMBO J.* 12, 3753–3760
3. Singleton, D. R., Wu, T. T., and Castle, J. D. (1997) *J. Cell Sci.* 110, 2999–3007
4. Laurie, S. M., Mixon, M. B., and Castle, J. D. (1991) *J. Biol. Chem.* 266, 19110–19117
5. Brumell, J. H., Volchuk, A., Sengelov, H., Borregaard, N., Cieutat, A. M., Bainion, D. F., Grinstein, S., and Klip, A. (1995) *J. Immunol.* 155, 5750–5759
6. Miligram, S. L., Kho, S. T., Martin, G. V., Mains, R. E., and Epper, B. A. (1997) *J. Cell Sci.* 110, 695–706
7. Fischer, Y., Thomas, J., Sevilla, L., Munze, P., Becker, C., Holman, G., Kekza, J., Palacin, M., Testar, X., Kammermeier, H., and Zorzano, A. (1997) *J. Biol. Chem.* 272, 7085–7092
8. Cameron, R. S., Cameron, P. L., and Castle, J. D. (1986) *J. Cell Biol.* 103, 1299–1313
9. Wu, T. T., and Castle, J. D. (1998) *Mol. Biol. Cell* 9, 1661–1674
10. Guo, Z., Turner, C., and Castle, J. D. (1998) *Cell* 94, 537–548
11. Sinzuk, J., Frisch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Rosahl, T. W., Spillane, D., Missler, M., Herz, J., Selig, D. K., Wolf, J. R., Hammer, R. E., Malenka, R. C., and Sudhof, T. C. (1995) *Nature* 375, 488–493
13. Wen, G., Leeb, T., Hui, D., Baumgartner, B. G., Robie, A., Hameister, H., and Brenig, B. (1998) *Nature Genet.* 9, 536–539
14. Geppert, M., Goda, Y., Hammer, R. E., Li, C., Rosahl, T. W., Stevens, C. G., and Sudhof, T. C. (1994) *Cell* 79, 717–727
15. McMahon, H. T., Bolshakov, V. Y., Janz, R., Hammer, R. E., Siegelbaum, S. A., and Gomperts, B. D. (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93, 4760–4764
16. Johnston, P. A., Jahn, R., and Sudhof, T. C. (1989) *J. Biol. Chem.* 264, 1268–1273
17. Alvarez de Toledo, G., Fernandez-Chacon, R., and Fernandez, J. M. (1993) *Nature* 363, 554–558
18. Fernandez-Chacon, R., and Alvarez de Toledo, G. (1995) *FEBS Lett.* 363, 221–225
19. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) *Pflugers Arch.* 391, 89–100
20. Lindau, M. (1991) *Q. Rev. Biophys.* 24, 75–101
21. Fyke, E. M., Talei, K., Walsh-Sulimena, C., Geppert, M., Jahn, R., De Camilli, P., and Sudhof, T. C. (1993) *J. Neurosci.* 13, 4997–5007
22. Fernandez, J. M., Neher, E., and Gomperts, B. D. (1984) *Nature* 312, 453–455
23. Almers, W., and Neher, E. (1987) *J. Physiol.* (London) 386, 265–277
24. McNex, D. J., Alvarez de Toledo, G., and Fernandez, J. M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 7804–7808
25. Alvarez de Toledo, G., and Fernandez, J. M. (1990) *J. Cell Biol.* 110, 1033–1039
26. Lindau, M., and Gomperts, B. D. (1991) *Biochem. Biophys. Acta* 1071, 429–447
27. Oberhauser, A. F., and Fernandez, J. M. (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93, 14349–14354

---

\[2\] R. Fernández-Chacón and T. C. Südhof, unpublished observation.