concentrated at the TGN. Although the association of CALM and syntaxin2 was not detected in lysates of wild-type cells, we did observe a rather irregular localization of syntaxin2 in *SMAP2*(–/–) cells. Such alterations in the composition of proacrosomal vesicles might be related to the apparent defect in fusion of proacrosomal vesicles into an acrosomal structure that resulted in the generation of nonfused, segmented acrosomes. The present study therefore suggests a scenario in which the budding and fusion of proacrosomal vesicles are linked by the recruitment of specific proteins to the vesicles.

Malformation of the acrosome causes secondary effects on the manchette (Kierszenbaum *et al.*, 2004; Pierre *et al.*, 2012). In particular, the manchette cannot be localized to the perinuclear ring; thus the manchette–acrosome connection becomes disorganized, and spermatid–Sertoli cell adhesion is perturbed (Kierszenbaum *et al.*, 2007). In *SMAP2*-deficient spermatids, the manchette was sometimes mislocalized, and the ectoplasmic specializations from the Sertoli cells were not formed properly, or the anchoring of the acrosome to the nucleus appeared weak, thus allowing invagination of the Sertoli cells into the spermatid. Thus, in *SMAP2*-deficient spermatogenesis, both primary defects in proacrosomal vesicle formation and secondary defects in manchette attachment likely contribute to the abnormal globozoospermic morphology (Kierszenbaum and Tres, 2004; Kierszenbaum *et al.*, 2011; Fujihara *et al.*, 2012).

Endocytosis of cell membrane proteins also contributes to the formation of the acrosome (Moreno and Alvarado, 2006; Berruti *et al.*, 2010; Rainey *et al.*, 2010; Paiardi *et al.*, 2011). Our previous study using cell lines showed that SMAP2 is located on early endosomes (Natsume *et al.*, 2006). Therefore we cannot exclude the possibility that some defects in retrograde traffic from early endosomes to the TGN might also be involved in the genesis of the globozospermia seen in *SMAP2*(–/–) mice.

There are several mouse models reported to exhibit abnormalities in proacrosomal vesicle formation from the TGN. In gene-targeted mice with knockouts of HIV-Rev-binding protein (*Hrb*), Golgi-associated PDZ and coiled-coil motif-containing protein (*GOPC*), Hook homologue 1 (*Hook1*), or protein interacting with C kinase 1 (*Pick1*), acrosome formation is defective. All of these molecules are involved in the transport of proacrosomal vesicles from the TGN (Kang-Decker *et al.*, 2001; Yao *et al.*, 2002; Moreno *et al.*, 2006; Xiao *et al.*, 2009; Kierszenbaum *et al.*, 2011). Among these knockouts, Hrb belongs to an Arf GAP family, as does SMAP2. *Hrb*-deficient male mice exhibit globozoospermia, but the details of the phenotype appear distinct from *SMAP2*-deficient spermatogenesis. In *Hrb*-deficient mice, the number of mature sperm is extremely low, some sperm possess multiple nuclei due to a defect in meiosis, and other sperm possess multiple flagella due to an increased number of centrioles (Juneja and van Deursen, 2005). These features were not observed in *SMAP2*-deficient spermiogenesis. Furthermore, Hrb plays a role distinct from SMAP2, in that the Hrb protein is located on the cytoplasmic side of the proacrosomal vesicles and functions in their fusion. Thus, although both SMAP2 and Hrb are Arf GAPs and their deficiencies cause globozoospermia, the two proteins appear to exert nonoverlapping, unique functions during spermatogenesis.

MATERIALS AND METHODS

Generation of *SMAP2*-targeted mice

A lambda phage clone encompassing exon 1 of *SMAP2*, which contains the methionine start codon, was isolated from a 129/SvJ genomic library (Stratagene, Santa Clara, CA). A targeting vector was constructed in which a fragment containing the *LacZ* and *PGK-neo*^{R} sequences was inserted into the *Kpn*I site of exon 1, and a *diphtheria toxin-A*–encoding fragment was ligated at the 5′ end. The vector was linearized by *Not*I digestion and electroporated into E14 ES cells. Of 610 G418-resistant clones, five were judged to have undergone proper homologous recombination based on PCR and Southern blot analysis of genomic DNA. Procedures for PCR and Southern blotting were as previously described (Wong *et al.*, 2010). Cells with a normal karyotype were selected using a Colcemid treatment method. Three ES cell clones were injected into blastocysts, and the chimeric males that were produced were mated with C57BL/6J female mice. Finally, two lines of *SMAP2*(+/–) founder mice were established that possessed the *SMAP2*-targeted allele in the germline. PCR primers used for genotyping were as follows. To detect the wild-type allele, the forward and reverse primers were 5′-CACTCGGGGTCAAGTGTGCG-3′ and 5′-CCAGAACCCCCTCCCCACTC-3′, respectively. To detect the targeted allele, the forward and reverse primers were 5′-CGCCTTCTATCGCCTTCTTGACG-3′ and 5′-CTTTCCGCCTCAGAAGCCATAGAG-3′, respectively.

In vitro fertilization and intracytoplasmic sperm injection

For in vitro fertilization, mature oocytes were collected from oviducts of B6D2F1 female mice that had been superovulated by the injection of 5 IU each of equine and human chorionic gonadotrophin. Sperm were collected from the epididymides of *SMAP2*(+/–) or (–/–) male mice. In each experiment, oocytes from two females were incubated with sperm from one male at a concentration of 200 sperm/μl. Fertilization was confirmed by development to the two-cell stage by 24 h after insemination.

For intracytoplasmic sperm injection, germ cells were collected from seminiferous tubules of *SMAP2*(+/–) or (–/–) testes. Elongated spermatids that were at differentiation steps 9–11 were picked up and injected into oocytes using a piezo-driven micromanipulator (PrimeTech, Tsuchiura, Japan). Oocytes that survived injection were cultured in a potassium simplex optimized medium, and those that developed to the two-cell stage were transplanted into oviducts of pseudopregnant ICR female mice. The offspring were counted at full term, namely, on day 19.5, by caesarian section or after natural delivery.

Transplantation of spermatogonial stem cells

Four-week-old WBB6F1-W/W^v mice that were devoid of spermatogenic cells were purchased from Japan SLC (Hamamatsu, Japan) and used as recipient animals. Spermatogonial stem cells were prepared from *SMAP2*(–/–) testes by a two-step enzymatic digestion technique as described previously (Ogawa *et al.*, 1997). A volume of 10 μl of spermatogenic cells, (2–3) × 10^7 cells/ml, was introduced into the seminiferous tubules of the WBB6F1-W/W^v mouse testis. As a control experiment, spermatogonial stem cells of *SMAP2*(+/+) mice were transplanted into *SMAP2*(–/–) mice. For recipient preparation, busulfan (44 mg/kg body weight) was injected intraperitoneally into 3-wk-old *SMAP2*(–/–) male mice. After 6 wk, the busulfan-treated testes were devoid of endogenous spermatogenesis. Spermatogonial stem cells were prepared from the testes of pCXN-EGFP transgenic, *SMAP2*(+/+) male mice (Okabe *et al.*, 1997) and introduced into the seminiferous tubules of the busulfan-treated *SMAP2*(–/–) mice.

At 3 mo posttransplantation, mice were killed and testes were removed. The seminiferous tubules were dissected out of the testes, and sperm released from the tubules was observed with an Olympus BX50 microscope (Olympus, Tokyo, Japan) using DIC optics. The tubules were transferred into a fixative containing 2.5% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and processed for microscopic observation.

Histology and immunofluorescence detection

Testes were fixed in Bouin's solution, embedded in paraffin, and sectioned. Deparaffinized sections were stained with hematoxylin and eosin, toluidine blue, or PAS, according to standard procedures. Some testes were fixed in 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS), immersed in 20% (wt/vol) sucrose in PBS, frozen in an optimal cutting temperature compound, cryostat sectioned, and processed for immunofluorescence. For some samples, germ cells were liberated as single-cell suspensions from testes or epididymides, centrifuged onto slide glasses, fixed, and processed for immunofluorescence. The following antibodies were used: anti-SMAP2 (Sigma-Aldrich, St. Louis, MO), anti-γH2AX (Trevigen, Gaithersburg, MD), anti-TGN38 (AbD Serotec, Raleigh, NC), anti–clathrin heavy chain (Affinity BioReagents, Golden, CO), anti-syntaxin6 (BD Transduction Labs, Lexington, KY), anti-GM130 (BD Transduction Labs), anti-sp56 (QED Bioscience, San Diego, CA), anti-syntaxin2 (Abcam, Cambridge, MA), anti-CALM (Santa Cruz Biotechnology, Santa Cruz, CA), and appropriate fluorophore-conjugated secondary antibodies. Rhodamine-conjugated PNA and MitoRed were obtained from Vector Laboratories (Burlingame, CA) and Dojindo (Rockville, MD), respectively. Fluorescence was observed using a BZ9000 fluorescence microscope (Keyence, Osaka, Japan). The Pearson's r quantifying the colocalization between images was calculated using the JACoP plug-in in ImageJ (National Institutes of Health, Bethesda, MD).

Electron microscopic observations

Mice were killed and perfused with a fixative containing 2.5% (wt/vol) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Testes and epididymides were removed and treated with the same fixative at 4°C overnight. Tissues were cut into small pieces, rinsed, and postfixed in 1% (wt/vol) OsO_4 in distilled water for 1 h. The samples were dehydrated using increasing concentrations of ethanol and embedded in Epon 812. The plastic-embedded tissues were sectioned using an ultramicrotome (model Ultracut E; Reihert-Jung, Vienna, Austria) and stained with uranyl acetate and lead citrate. The samples were observed using a JEOL 1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

Northern blot, immunoblot, and immunoprecipitation analyses

RNA was isolated from cells/tissues of C57BL/6J mice using TRIzol (Life Technologies, Carlsbad, CA). When necessary, germ cells from testes were fractionated into various differentiation stages by centrifugal elutriation (Barchi *et al.*, 2009). For Northern blots, 10 μg of total RNA was separated in a 1% (wt/vol) formaldehyde/agarose gel and transferred to a Hybond membrane (Amersham, Piscataway, NJ), according to the manufacturer's instructions. The filter was hybridized with a radiolabeled *Sph*I fragment of *SMAP2* cDNA.

Protein lysates of cells/tissues were prepared with a Polytron homogenizer using a buffer containing 9 M urea, 2% (vol/vol) Triton X-100, and 1% (wt/vol) dithiothreitol. The lysates were centrifuged at 13,000 rpm for 15 min, and the supernatant was collected. Lithium dodecyl sulfate was added to a final concentration of 2% (wt/vol), and the solution was sonicated. The protein concentration was determined using a Bio-Rad (Hercules, CA) protein assay kit. Protein samples (20 μg) were separated on an 8% SDS–PAGE gel and electrotransferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). Immunoblotting was performed using anti–β actin (Sigma-Aldrich), anti-SMAP2, anti-CALM, or anti-syntaxin2 antibodies and an ECL reagent (Amersham). Some lysates were first immunoprecipitated, as described previously (Natsume *et al.*, 2006), and the precipitates were then blotted using one of the antibodies described.

Statistical analyses

Statistical significance was evaluated using a two-tailed Student's t test, and differences of $p < 0.05$ were considered statistically significant.

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