Mutations in a new scaffold protein Sans cause deafness in Jackson shaker mice

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The Jackson shaker (js) mouse carries a recessive mutation causing phenotypes such as deafness, abnormal behavior (circling and/or head-tossing) and degeneration of inner ear neuroepithelia. Two alleles have been identified so far, the original js and jsseal. A contig of three BAC clones was isolated by positional cloning. Two of the clones rescue the js phenotype by BAC transgenesis. Analysis of transcripts in an overlapping region of the two clones revealed a gene encoding a new scaffold-like protein, Sans, that showed mutations in the two js mutants. One was a guanine nucleotide insertion in the original js allele and the other a 7-base insertion in the jsseal allele. Both insertions are predicted to inactivate the Sans protein by frameshift mutations resulting in a truncated protein lacking the C-terminal SAM domain. Cochlear hair cells in the js mutants show disorganized stereocilia bundles, and Sans were highly expressed in inner and outer hair cells of cochlea. The existence of major motifs, ankyrin repeats and a SAM domain suggests that Sans may have an important role in the development and maintenance of the stereocilia bundles through protein–protein interaction.

INTRODUCTION

Over 200 mouse mutations affecting the inner ear have been mapped in the mouse genome (MRC Institute of Hearing Research web site at www.ihr.mrc.ac.uk/hereditary/MutantsTable.shtml). Recently, a number of mouse mutations have been identified by positional cloning, which is providing a rich source of models for human deafness. Indeed, the genetic analysis of those mutations has proven valuable for the identification of genes causing human deafness, such as myosin VIIa (1–3), myosin XV (4,5), cadherin 23 (6,7) and protocadherin 15 (8,9), etc. Moreover, these hereditary hearing-loss genes identified through human and mice are useful for determining the mechanisms underlying both normal and pathologic processes and for understanding the normal process of auditory development and sensory transduction.

One such mouse model is Jackson shaker (js). Homozygous js mice exhibit the typical circling, head-tossing, hyperactive behavior and deafness which are caused by the degeneration of the neurosensory epithelium of the inner ear (10,11). Two js alleles have been identified: js, a spontaneous allele that arose in the inbred strain A/J, and jsseal, a spontaneous mutation in the strain C3H/HeN (12,13). Classical genetic mapping studies have positioned js to the distal part of chromosome 11. Also of significant interest is the finding that Usher syndrome type IG (USH1G) and two dominant loci have been mapped to the human homologous region on chromosome 17q24–25 (14–16). The USH1G locus is for human disorder associated with...
deafness and progressive visual loss, known as Usher syndrome type IG, and the DFNA20 and DFNA26 are for nonsyndromic deafness.

We report here that the js gene encodes a new scaffold protein, termed Sans (scaffold protein containing ankyrin repeats and SAM domain). We also show that Sans is expressed specifically within the neurosensory epithelium. The expression pattern, combined with the defects observed in js mice, identifies Sans as a molecule required for normal hearing.

RESULTS

Figure 1A shows a genetic map of the js locus on mouse chromosome 11 and Figure 1B represents a physical map consisting of three overlapping BAC clones: 138E1, 212O4 and 150N14. The js locus was located within a non-recombinant interval of ~300 kb flanked by two SSLP markers, D11Rin3 and D11Rin123, where 13 expressed genes existed as identified by cDNA selection (data not shown). To narrow down the js interval further, we performed phenotype rescue by BAC-transgenesis with the three clones. Four transgenic (Tg) lines were established: TG-212-1, TG-212-2, TG-138 and 150 (Fig. 2A and B and Table 1). One Tg line, TG-212-1, showed normal ABR (auditory brainstem response), and behaviors (Fig. 2C, Table 1). The recovery of cochlear defects by BAC transgenesis were confirmed by histological analysis of 6-week-old wild-type, js and TG-212-1 mice (Fig. 2D). At 4 weeks of age, TG-212-1 showed normal morphologies of the cochlea, whereas there is already complete loss of inner and outer hair cells within the organ of Corti and a low density of spiral ganglion cells in js mice. On the other hand, js mice exhibit disorganization of stereocilia bundles in hair cells as the major defect (11,12). To confirm more precisely, we performed F-actin staining using the rhodamine-conjugated phalloidin probe to examine whether the js phenotype, mainly disorganization of the stereocilia bundles was rescued by BAC transgene (Fig. 2E–G). In the js mice, degeneration of the stereocilia bundles, of inner and outer hair cells was apparent at 4 weeks (Fig. 2E). Compared with js mice, the stereocilia bundles of TG-212-1 mice exhibited normal phenotype. The blunt V-shaped stereocilia bundles of TG-212-1 mice can be clearly seen on the outer hair cells (Fig. 2F), and also rescued
as normal hair bundles of inner hair cells (Fig. 2G). Two other lines, TG-212-2 and TG-150, also showed similar normal phenotypes. Because the js phenotype was rescued by the two BAC clones, 212O4 and 150N14, but not by 138E1, the js gene is probably located in the overlapping region of 72.2 kb between 212O4 and 150N14 (Fig. 1B).

This 72.2 kb region was completely sequenced and putative exons were found with the aid of the Grail and BLAST search tools.

**Figure 2.** BAC transgene correction of Jackson shaker. (A) Identification of transgenic mice by Southern blot analysis using a probe specific for the BAC vector. (B) PCR typing of the four transgenic lines produced. The SSLP marker used was D11Rin130 on the Srams gene. BAC transgenes were derived from 129/Sv (Research Genetics/Invitrogen), and D11Rin130 is polymorphic between js and 129/Sv mice. Therefore, the transgenic lines rescued by BAC clones, TG212-1, TG212-2 and TG-50, possessed the 129/Sv allele of the SSLP marker. (C) ABRs from the js mouse and a js-tg mouse from TG212-1. The five major peaks (labeled I, II, III, IV and V) of the averaged waveform were detectable up to 20 dB peSPL in a tg mice, while no waveform was recorded at the highest stimulus level of 90 dB peSPL in a deaf mouse. (D) Cochlear histology of 6-week-old wild-type, js and js-tg mice. The organ of Corti (Co) of middle turn of the cochlear duct is shown. Black arrows indicate degeneration of the Co and a low density of spiral ganglion cells (SGC) in js and js-tg mice. These phenotypes of Co and SGC were confirmed to be completely rescued in js-tg mice. (E–G) Phenotype of stereocilia bundles on inner hair cells (IHC) and outer hair cells (OHC) in 4-week-old wild-type, js and js-tg mice. To visualize the F-actin-rich stereocilia bundles on the hair cells, the cochlear duct was stained with a rhodamine-conjugated phalloidin, and then analyzed with laser scanning confocal microscopy. The presence of normal stereocilia on IHC and OHC in js-tg mice was demonstrated. The normal stereocilia bundles were clearly observed in higher magnification (F, G). White arrows indicate disorganized stereocilia of js mice (F). The blunt V-shaped stereocilia bundles of js-tg mice can be clearly seen on the outer hair cells (F). The stereocilia on inner hair cells in js-tg also were recovered as normal phenotype (G). Scale bar = 20 μm.
programs (Fig. 1C). RT–PCR analysis of these exons grouped them into five expressed genes, and the existence of each of these was confirmed by RT–PCR, using 5′- and 3′-RACE with mRNA from the brain and inner ear (Fig. 1C). The five genes represent novel genes with unknown function. To screen for mutation(s) associated with the js phenotype, we performed genomic sequencing of js/js, js/seal/js/seal and wild-type mice and found mutations in one of the genes. Its full-length cDNA clone (3202 nucleotides in all) contained one open reading frame encoding a polypeptide of 461 amino acids. The mutations found were a single guanine insertion in a stretch of sequence with six guanines at the nucleotide positions 675–680 in the js allele (Fig. 3B), and a 7 bp (GTCAGCA) insertion at nucleotide position 896–897 in the js/seal allele (Fig. 3C). Both insertions are predicted to cause frameshift mutations that result in truncation of the peptide chains by newly generated stop codons at amino acid position 245 in the js mutant and at amino acid position 350 in the js/seal mutant (Fig. 3D). Database searches using BLAST, PROSITE and PROFIFE programs revealed that the complete protein sequence possesses ankyrin (Ank) repeats and a sterile alpha motif (SAM) as major functional motifs near the N- and C-termini, respectively. However, the protein structure differed from that of any other known genes, and therefore we named this novel gene Sans.

Figure 2 continued.

Table 1. Rescue of the js phenotype by BAC transgenesis

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Injected BAC</th>
<th>TG-mouse used (js/js-tg)</th>
<th>Phenotype</th>
<th>Behavior</th>
<th>Hearing test</th>
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<td>12</td>
<td>2</td>
<td>js</td>
<td>Normal</td>
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<tr>
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<td>12</td>
<td>Normal</td>
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Each transgenic line was derived independently from one founder (all male mice). These founders were mated to a js/+ female. The TG-mouse indicates the number of js/js genotype and transgene positive progeny. Behavior of mice detected by circling (js) or normal. Hearing test was performed by ABR analysis. ND, not done.
C-terminal SAM domain. The frameshifts may also lead to functional inactivation through rapid degradation of mRNA by a nonsense codon-mediated mRNA decay mechanism, although js mice appear to have normal levels of Sans mRNA (data not shown).

To study the expression of the Sans gene in the inner ear, we amplified a gene-specific RT-PCR product from a cDNA synthesized from mouse cochlear mRNA, and found high levels of expression in the cochlea. In other adult tissues, we also detected high levels of expression in testis, cerebellum and eye, and low levels of expression in brain, thymus and spleen (Fig. 4A). Low-level expression was also detected in E17.5 embryos but not in embryos earlier than E15.5 (Fig. 4A). Tissue localization of the message for this protein was determined by in situ hybridization of inner ear sections from newborn mice using an antisense RNA probe. Significant signals were detected in the neurosensory epithelium of inner ear cochlea (Fig. 4B) and sacculus (Fig. 4C), especially in inner and outer hair cells (Fig. 4D). In contrast, no signals were detected using a control sense probe (Fig. 4E, F and G).

**DISCUSSION**

Our data show that the deafness and abnormal behavior of js mutants are the result of mutations in the gene that encodes the Sans protein. Based on the following findings, we propose that the Sans is a new deafness gene. First, the js phenotype was rescued by the two BAC clones including Sans gene (Fig. 1B and C, Table 1). Second, the two js alleles of the Sans gene had frameshift mutations that altered the sequence of the C-terminal SAM domain (Fig. 3D). Third, Sans mRNA is localized to the neurosensory epithelium of the inner ear and primary sites of degeneration noted in js mice (Fig. 4B–D).

Although the function of the Sans protein is currently unknown, one can hypothesize its function from the presence of the major motifs, the Ank repeats and the SAM domain (Fig. 3D). The Ank repeats are tandem repeats of about 33 amino acids constituting a β-hairpin and two α-helices that potentially provide a site for protein–protein interaction (17,18). The SAM domain is a protein module of ~70 amino acids found in a variety of signaling molecules, which mediates the homotypic and heterotypic dimerization of their proteins.
There are a few known proteins that contain a combination of these Ank repeats and SAM domains, e.g. SHANK family (22), CASK-interacting proteins (23), TANK (24) and GASZ (25). For example, the SAM domain of the SHANK protein seems to multimerize as homomers or heteromers to allow cross-linking of several proteins at post-synaptic sites of the brain excitatory synapses (22). It is therefore speculated that SHANKs are candidate master organizers of post-synaptic specialization. Likewise, Sans might also function as an anchoring/scaffolding protein in hair cells.

Several mouse mutations that cause deafness and abnormal behavior (circling/head tossing) are known. These include js mice, which display abnormal morphologies in stereocilia of the neurosensory epithelia. However, their mutants show distinctive stereocilia defects, for example, myosin VIIa (shaker-1), cadherin 23 (waltzer) and protocadherin 15 (Ames waltzer) mutants show disorganized stereocilia bundles (6,8,26), myosin XV (shaker-2) and espin (jerker) mutants show short stereocilia (4,27), and myosin VI (Snell’s waltzer) mutant shows early fusion of stereocilia (28).

In previous study using scanning electron microscopy, the stereocilia of Sans (js) mutant display an irregular arrangement, and then this disorganization of the stereocilia bundle increases in older mice (11,12), which is essentially consistent with disorganization.

Figure 4. Expression profile of Sans. (A) Expression in adult and embryonic tissue. cDNA from the indicated tissues was used to amplify a 850 bp Sans-specific PCR product (top). cDNA integrity was confirmed with a 1 kb Gapdh control band (bottom). (B–D) In situ hybridization of the mouse cochlea (newborn) using a mouse Sans antisense probe. The antisense probe labels the neurosensory epithelium (NE) of cochlea (B) and saccule (C), and especially labels inner hair cells (IHC) and outer hair cells (OHC) of cochlea (D). (E–G) Cross section of the cochlea showing the absence of labeling with the sense probe. SCV, scala vestibuli, RM, Reissner’s membrane, CD, cochlear duct, Co, organ of Corti.
of stereocilia bundles seen, especially in planar polarity mutants such as shaker-1, waltzer and Ames waltzer. On the other hand, because the hair cells of these mutants at the early stage showed no obvious abnormalities in the cell body or cuticular plate (6,8,11,26), their responsible genes may have a role in the development and maintenance of the stereocilia bundles. Interestingly, products of these genes are co-localized in the cochlear hair cells (9,29,30), and contain protein–protein interaction domains (3,9,30). Indeed, the C-terminal FERM domain of myosin VIIa binds to a novel transmembrane protein, vezatin (31), and cadherin 23 form a protein complex with harmonin in stereocilia bundles of hair cells (30). Therefore, Sans may mediate these protein complex through its protein–protein interaction domains. In addition, defects in the formation of protein complex including Sans may disrupt stereocilia bundles in js mice. To clarify these issues, we attempted protein–protein interaction analyses such as immunohistochemistry, yeast two-hybrid assay, immunoprecipitation, etc.

Recently, the major involvement of the Sans gene has been discovered in three families of Usher type IG syndrome (USH1G) (Weil et al., personal communication). USH1 is characterized by severe to profound congenital sensorineural deafness, constant vestibular dysfunction and prepubertal onset retinitis pigmentosa (32). These lines of evidence in both mice and humans suggest the possibility that the Sans gene can act as a major contributor to hearing and balance impairment. Incidentally, although Sans is detected expression of high level in the eye as shown by RT–PCR (Fig. 4A), our preliminary findings suggest the absence of gross physiological abnormalities in the retina of js mice (unpublished data). Possibly, additional studies may reveal the phenotype in js mice, as has been the case with shaker-1 mutant (33). USH1 is the most common subtype for which seven loci (USH1A-G) have been reported (14,31). The genes mutated at four of these loci besides Sans already have been identified: myosin VIIa at USH1B, harmonin at USH1C, cadherin 23 at USH1D and protocadherin 15 at USH1F (2,7,9). As mentioned above, in mice, mutations in myosin VIIa, cadherin 23 and protocadherin 15 cause deafness and abnormal behavior in shaker-1, waltzer and Ames waltzer mutants, respectively (1,6,8). These findings, combined with phenotypes of these mutants, including js mice, open up a new pathway in the inner ear.

**MATERIALS AND METHODS**

**Mice**

C57BL/6J-js/js mice were obtained from The Jackson Laboratory. The js mutations arose spontaneously in the A/J strain at The Jackson Laboratory and were then maintained in the C57BL/6J-congenic strain. The js^eal^ allele arose spontaneously in the C3H/HeN strain that had been maintained at the Laboratory of Animal Science, Institute of Medical Science, University of Tokyo.

**Linkage and physical mapping**

Linkage maps for js and js^eal^ were made by intersubspecific backcrossing using progeny derived from mating of (MSM/Ms × C57BL/6J/js/js)F₁ × js/js and (MSM/Ms × C3H/HeN-js^eal^/js^eal^)F₁ × js^eal^/js^eal^. A total of 1916 progeny were typed for the markers D11Mit128, D11Mit214 and D11Lpk4. The recombinants between these markers were then typed using markers D11Mit257, D11Mit338 and D11Rin3 (PCR primers; 5'-TTC CAC GAA GGT CTG CAT GGA CT-3' and 5'-TTCC AAA GAA AAT CAA CC-3') and D11Rin123 (5'-ACT CAC AGT GAA TGG CTT ACA GCT C-3' and 5'-CTG AGA GAT GGT TCA GCC ACT AAT G-3').

The mouse BAC clones were isolated from a murine CITB BAC library, which has been derived an inbred line of mice 129/SvJ (Research Genetics/Invitrogen). A BAC-based physical map of the area surrounding the js region was generated by chromosomal walking using STS markers developed from BAC end sequences.

**BAC transgenesis**

Closed circular forms of BAC DNAs were isolated using an alkaline lysis and cesium chloride gradient ultracentrifugation protocol (34). Alter overnight dialysis, DNA was diluted to a concentration of 10 ng/μl in TE buffer [10 m M Tris–HCl (pH 7.5), 0.1 mM EDTA]. The isolated cc BAC DNA was microinjected following a standard technique for cDNA or genomic DNA clones into pronucleus-stage oocytes isolated from either B6-js/js or B6 females. Transgenic mice were screened both by PCR and Southern blot analysis of genomic DNA prepared from auricle or tail biopsies.

**ABR threshold measurements**

To measure the auditory brainstem responses (ABR), we used at least four individuals for each group, e.g. C57BL/6J, C57BL/6J-js/js, and their transgenic mice at the age of 6 weeks. ABR were recorded with stainless steel needle electrodes inserted subcutaneously into the vertex (active), one side of the retroauricular region (inactive) and the opposite thigh (ground). The stimulus sound in peak equivalent sound pressure level (peSPL) of a tone pip consisting of 0.1 ms slopes, 1 ms duration, 70 ms repeat interval with a frequency of 10 kHz was delivered in a free field in an electronically shielded room. A tweeter (PT-RIII, Pioneer) was placed 10 cm in front of the external acoustic foramen. The stimulus sound pressure was corrected using a Bruel and Kjær type 2636 noise meter. A microcomputer (ER-2104, GE Marquet) was used to analyze the averaged waveform.

**Histological analysis**

Cochlea were dissected from four individuals each of B6-js/js and B6-js/js-tg at 6 weeks of age, fixed in 4% paraformaldehyde overnight, and then decalcified in 5% EDTA/PBS for 1 week. Alter decalcification, the tissues were dehydrated, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin.

Using rhodamine-conjugated phalloidin as described (35), histological examination of the cochlea at 4 weeks of age was undertaken.
Gene identification

BAC DNA was sequenced by the shotgun method. BAC212 and 150 were partially digested with Sau3AI and Rsal, and fractionated by agarose gel electrophoresis. Only fragments of 1–4 kb long were subcloned into a BamHI- and HindIII-digested and BAP-treated pUC118 vector (Takara). Plasmid DNA was prepared using the REAL Prep Kit (Qiagen), and the inserts were sequenced using a BigDye Terminator Kit (Applied Biosystems) and analyzed on a 377 sequencer and 3100 genetic analyzer (Applied Biosystems). The raw sequence data were aligned using the DNASIS program (Hitachi Software) and potential coding regions identified using the BLAST and Grail programs. The full-length cDNA was obtained using a combination of RT–PCR, 5' and 3'-RACE and by screening cochlear and brain cDNA libraries. 5'- and 3'-RACE were carried out using the Full RACE Core set (Takara) following the manufacturer's protocol.

Detection of js mutations

We amplified 12 genomic DNA fragments covering the 27 coding exons using a kit for long PCR (Takara) (see Fig. 1B). Direct sequencing of PCR products was carried out for genomic DNA of js homozygotes and heterozygotes, js\textsuperscript{r}eal homozygotes and heterozygotes, C57BL/6J, A/J and C3H/HeN mice. The following pairs of primers were used for amplification of the mutant region: MP2F, 5'-AAC CTG GAG TCT CTG CGC CTC ATT GTG-3', and MP5R, 5'-TCT GCC CAG TCT AGG GAG TTG AAG GAG-3', and the primer for sequencing: MP7F, 5'-TGA GCC GAC GCC TGC AGC ACA TGA C-3'.

RT–PCR and RNA in situ hybridization

Approximately 1 μg of DNase-pretreated total RNA prepared from 12 different mouse tissues and from embryonic tissues of five developmentally different stages was reverse-transcribed using the Omniscript RT kit (Qiagen). The cDNA was amplified for 30 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 1 min) using AmpliTaq Gold and a 9700 Thermocycler (Applied Biosystems). The products were subjected to agarose gel electrophoresis. Primers used for detection of \textit{Sans}-specific transcripts were: MP1F, 5’-AAC TCT GGG CCC ATG ATG AAC CAG CAG TAT C-3' and MP7R, 5’-ACG CTGT CCT CGT CCG AGA GGA AC ATG-3'.

For \textit{in situ} hybridization, digoxigenin-labeled sense and antisense RNA probes were synthesized using a DIG RNA Labeling kit (Roche Molecular Biochemicals). The template was the coding sequence fragment of 664 bp (nucleotide positions 619–1283) that was cloned in the pDrive vector (Qiagen). Newborn mice were fixed in 10% neutral buffered formalin, dehydrated, and then embedded in paraffin, and sectioned at 4 μm. Sections were hybridized with the RNA probe and stained by NBT/BCIP. Counterstaining was performed by kernechtrot.

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