

## Report

# Mutations in the *VLGR1* Gene Implicate G-Protein Signaling in the Pathogenesis of Usher Syndrome Type II

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Usher syndrome type II (USH2) is a genetically heterogeneous autosomal recessive disorder with at least three genetic subtypes (USH2A, USH2B, and USH2C) and is classified phenotypically as congenital hearing loss and progressive retinitis pigmentosa. The *VLGR1* (*MASS1*) gene in the 5q14.3-q21.1 *USH2C* locus was considered a likely candidate on the basis of its protein motif structure and expressed-sequence-tag representation from both cochlear and retinal subtracted libraries. Denaturing high-performance liquid chromatography and direct sequencing of polymerase-chain-reaction products amplified from 10 genetically independent patients with USH2C and 156 other patients with USH2 identified four isoform-specific *VLGR1* mutations (Q2301X, I2906FS, M2931FS, and T6244X) from three families with USH2C, as well as two sporadic cases. All patients with *VLGR1* mutations are female, a significant deviation from random expectations. The ligand(s) for the *VLGR1* protein is unknown, but on the basis of its potential extracellular and intracellular protein-protein interaction domains and its wide mRNA expression profile, it is probable that *VLGR1* serves diverse cellular and signaling processes. *VLGR1* mutations have been previously identified in both humans and mice and are associated with a reflex-seizure phenotype in both species. The identification of additional *VLGR1* mutations to test whether a phenotype/genotype correlation exists, akin to that shown for other Usher syndrome disease genes, is warranted.

Usher syndrome is a clinically and genetically heterogeneous recessive disease with three clinical subtypes. The subtypes are differentiated on the basis of the severity of hearing loss and presence or absence of vestibular abnormalities. Patients with Usher syndrome type I (USH1) present with profound congenital hearing loss, vestibular areflexia (in most cases), and a progressive retinal degeneration, with impaired night vision and decreased visual fields diagnosed as retinitis pigmentosa (RP). Usher syndrome type II (USH2 [MIM 276900]) have a moderate-to-severe sensorineural hearing loss that is stable in most cases, normal vestibular function, and RP, whereas patients with type III (USH3) present

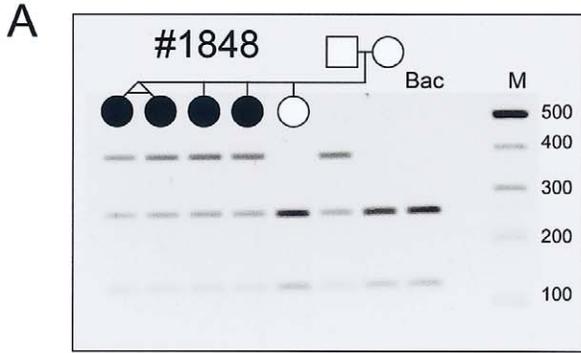
a moderate sensorineural hearing loss with progression to acquired deafness, progressive vestibular dysfunction, and RP. Currently, 11 loci have been identified for Usher syndrome (Kimberling et al. 1990, 1992; Kaplan et al. 1992; Smith et al. 1992; Sankila et al. 1995; Wayne et al. 1996, 1997; Chaib et al. 1997; Hmani et al. 1999; Pieke-Dahl et al. 2000; Mustapha et al. 2002); families that do not link to the known loci indicate additional genes exist. Of the 11 loci, 7 genes have been found with Usher syndrome mutations (Weil et al. 1995, 2003; Eudy et al. 1998; Bitner-Glindzicz et al. 2000; Verpy et al. 2000; Ahmed et al. 2001; Alagramam et al. 2001; Bolz et al. 2001; Bork et al. 2001; Joensuu et al. 2001).

Previously, we had localized the *USH2C* gene to a 20-cM region on chromosome 5q14.3–5q21.1 (Pieke-Dahl et al. 2000). Reference mRNAs in this linkage interval were obtained from the UCSC Genome Browser Web site and were prioritized for candidate-gene mutation screening. The very large G-coupled receptor gene (*VLGR1*) was considered a likely *USH2C* candidate (Burgess 2001; Staub et al. 2002), with EST representation in both human

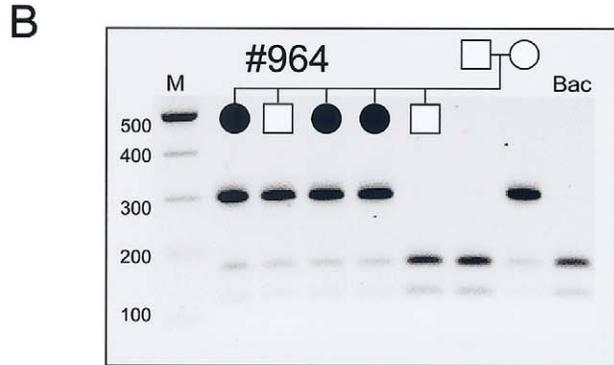
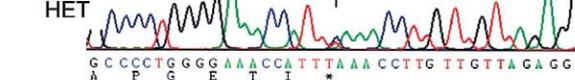
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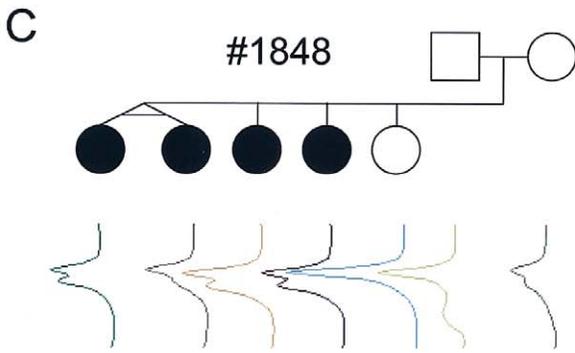
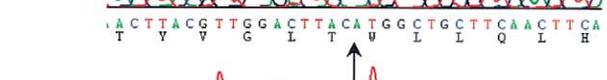
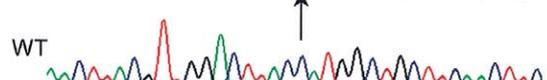
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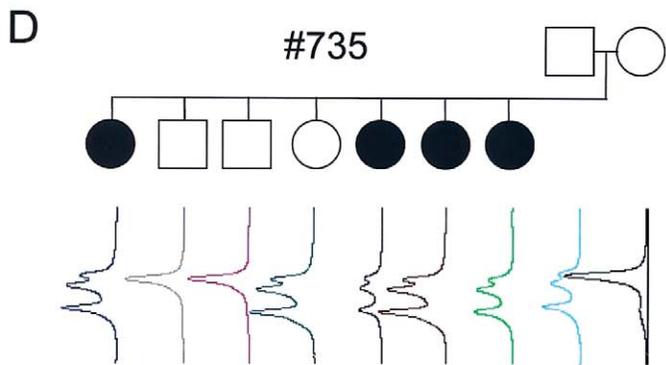
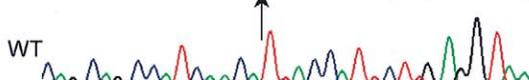
G C C C C T G G G G A A A C C A T T C A A A C C T T G T T G T T A G A G G  
 A P G E T I Q T L L L E V



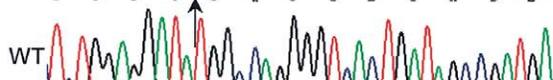
A A C T T A C G T T G G A C T T A C A T G G G C T G C T T C A A C T T C  
 T Y V G L T H A A S T S



C A G C A G C C A T C A A C A T T A C C A T T T C T T G A G G T A A  
 A A I N I T T I I E V T A K



T C T G G A G G A T A T G G C C A G G G G T C A C T G A T A G C C G A T G A  
 S G G V G Q G S L I A D E



fetal retina and cochlea (obtained from the National Eye Institute Gene Bank Web site) subtracted libraries. Use of the conserved domain architecture retrieval (CDART) tool on *VLGR1* (Geer et al. 2002) revealed a motif architecture similar to the cadherin superfamily of integral membrane proteins. This superfamily includes *CDH23* and *PCDH15*, which are the genes mutated in *USH1D* (MIM 601067) and *USH1F* (MIM 602083), respectively. In addition, *VLGR1* and the *USH2A* gene product usherin exhibit a high degree of similarity in their respective pentraxin (PTX) homology domains. On the basis of this evidence, we sought to implicate the *VLGR1* gene in the pathogenesis of *USH2*.

Denaturing high-performance liquid chromatography (DHPLC) and direct sequencing were used to detect mutations in PCR-amplified genomic DNA from 10 probands with *USH2C* (MIM 605472) for 90 *VLGR1* exons. The 10 affected probands were members of families consistent with genetic linkage to 5q14-21 (Pieke-Dahl et al. 2000). We had previously obtained informed consent and had extracted DNA from peripheral whole blood of the study subjects according to standard conditions (Puregene). Patients with a clinical diagnosis of *USH2* were ascertained through physician referral and self-referral. The study was conducted with the approval of the Boys Town National Research Hospital (BTNRH) institutional review board and met all ethical requirements and Health Insurance Portability and Accountability Act (HIPAA) standards. DNAs from an additional 152 unrelated patients with *USH2* without an identifiable *USH2A* mutation on chromosome 1q41 were used to screen for specific *VLGR1* mutations identified from either the mutation screen of the 10 probands with *USH2C* or from those *VLGR1* mutations reported elsewhere (Nakayama et al. 2002).

Genomic *VLGR1* PCR primer pairs were designed and synthesized commercially (sequences available upon request [Integrated DNA Technologies]). PCR used 20 ng genomic DNA, 0.2  $\mu$ M primer, 2.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP, in 1  $\times$  PCR buffer with 0.5 units AmpliTaq

Gold (PE Biosystems) in 25  $\mu$ l total volume. The initial PCR cycle was 95°C for 8 min and 30 s, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final step of 70°C for 10 min. BAC clones spanning the *VLGR1* genomic interval (CTD-2034A10, exons 1–4; RP11-29K14, exons 2–42; RP11-62E10, exons 18–73; RP11-3B6, exons 51–83; CTD-2001K4, exons 84 and 85; CTD-2266L18, exons 86 and 87; RP11-414H23, exons 88–90) were identified and used as a control template to evaluate PCR-product heteroduplexes that were separated by DHPLC; these clones were also used in direct PCR sequence analysis.

Three human *VLGR1* mRNA isoforms are expressed (*VLGR1a*, *VLGR1b*, and *VLGR1c*), of which only *VLGR1b* and *VLGR1c* isoforms are expressed in mouse (McMillan et al. 2002) (fig. 2). Of 72 nucleotide changes discovered in *VLGR1* (see table A [online only]), 4 mutations would prematurely terminate *VLGR1a* and *VLGR1b* protein translation (fig. 1). The *VLGR1* 6901C→T (Q2301X) and 8716-17insAACA (I2906FS) mutations were found in repulsion exclusively in the members of family 1848 who were affected with *USH2C* (fig. 1a and 1c). Family 964 (with *USH2C*) was found to segregate a maternal *VLGR1* 8790delC (M2931FS) mutation (fig. 1b). A paternal 19-bp deletion (18732–18750del19bp; T6244X) segregates appropriately with the phenotype in family 735 (fig. 1d). Families 735 and 964 have been shown elsewhere to genetically link to the 5q14.3-q21 region, with all affected siblings sharing family-specific 5q 14.3-q21 haplotypes (Pieke-Dahl et al. 2000). No other potential *VLGR1* variant was found associated with the maternal allele of family 735; however, a paternal IVS75-15G→A mutation absent in 190 control chromosomes was found segregating with the phenotype in family 964 (data not shown). It remains to be determined whether and how IVS75-15G→A affects *VLGR1* expression. Screening for *USH2C* mutations in 152 patients with *USH2* revealed the Q2301X mutation in two other unrelated patients with sporadic *USH2*. Table 1 presents a clinical summary of all patients

**Figure 1** Segregation of *VLGR1* mutations Q2301X, M2931FS, I2906FS, and T6244X in families 735, 964, and 1848 (with *USH2C*). A, Paternal inheritance of Q2301X in family 1848 is shown by agarose-gel electrophoresis of exon 31 PCR product *XmnI* digests. Q2301X mutation is a 6901 C→T transition, 33 bp on the 3' end of the alternate exon 31 splice donor, affecting only the *VLGR1b* mRNA isoform (fig. 2a). Shown here are sequence electropherograms from the heterozygous family 1848 proband (HET), an apparent Q2301X homozygote, and wild-type (WT) BAC clone RP11-29K14, with sequence and amino acid translation differences (arrows). The Q2301X homozygote was the result of a brother-sister incestuous union, whereas another singleton case was a Q2301X/occult heterozygote (data not shown; table 1). B, Maternal inheritance of M2931FS in family 964 is shown by agarose-gel electrophoresis of exon 39 PCR product *NcoI* digests. Sequence electropherograms of the WT and the cloned M2931FS allele show 8790delC in exon 39 (arrows). This deletion causes a 10-codon frameshift ending with a TAG stop encoded by the last 3 bases of exon 39, affecting *VLGR1b*. C, Maternal inheritance of I2906FS in family 1848 by DHPLC of exon 38 PCR products. Sequence comparison of the cloned I2906FS mutation shows an 8716-17insAACA (arrows) causing a frameshift of 5 codons and ending with a TGA stop 1 bp short of the 3' end of exon 38, affecting *VLGR1b*. D, Paternal inheritance of Y6244X detected by DHPLC of exon 89 PCR products. A 19-bp deletion brings a TAG stop codon immediately in-frame. Y6244X removes 63 amino acids from the COOH end of *VLGR1a* and *VLGR1b*. The putative maternal and paternal mutations in family 735 and family 964 have not been identified.

**Table 1****Clinical Summary of Patients with USH2C Harboring *VLGR1* Mutations**

Family No. and Individual No.	Severity of Hearing Loss	Age at RP Diagnosis (years)	Sex	RP Symptoms	<i>VLGR</i> Mutation(s)
735 <sup>a</sup> :					
1	Moderate-severe	27 <sup>b</sup>	Female	Typical	T6244X/occult
5	Moderate-severe	25	Female	NA	T6244X/occult
6	Moderate-severe	20	Female	NA	T6244X/occult
7 <sup>c</sup>	Moderate-severe	19	Female	NA	T6244X/occult
964 <sup>d</sup> :					
1	Moderate-severe	24	Female	Typical	M2931FS/occult
3	Moderate-severe	21	Female	Typical	M2931FS/occult
4	Moderate-severe	33	Female	Mild	M2931FS/occult
1127:					
1	Moderate-severe	42	Female	Typical	Q2301X/occult
1848 <sup>e</sup> :					
1	Moderate-severe	32 <sup>b</sup>	Female	NA	Q2301X/I2906FS
2	NA	29	Female	NA	Q2301X/I2906FS
3	NA	29	Female	NA	Q2301X/I2906FS
4	NA	21	Female	NA	Q2301X/I2906FS
2684 <sup>e</sup> :					
1	Moderate-severe	17	Female	NA	Q2301X/Q2301X

NOTE.—NA = not available.

<sup>a</sup> United States, country of origin.

<sup>b</sup> The RP diagnosis in the oldest child led to the earlier age at RP diagnosis in the other siblings.

<sup>c</sup> Reported that loud sounds caused nausea and, occasionally, dizziness.

<sup>d</sup> Sweden, country of origin.

<sup>e</sup> Consanguineous.

with USH2C who harbor *VLGR1* mutations. A total of five independent probands with USH2C and eight affected siblings were found to bear pathological *VLGR1* mutations. Two probands have pathological mutations identified in both alleles; three occult mutant alleles either have escaped detection by the methodology employed, lie in unscreened regions of the gene, or have been detected but not appreciated as disease alleles. None of the four *VLGR1* mutations was observed in 190 control chromosomes. These results establish *VLGR1* as the USH2C gene and implicate the protein, which is the largest cell surface receptor known (McMillan et al. 2002), in the pathogenesis of USH2C.

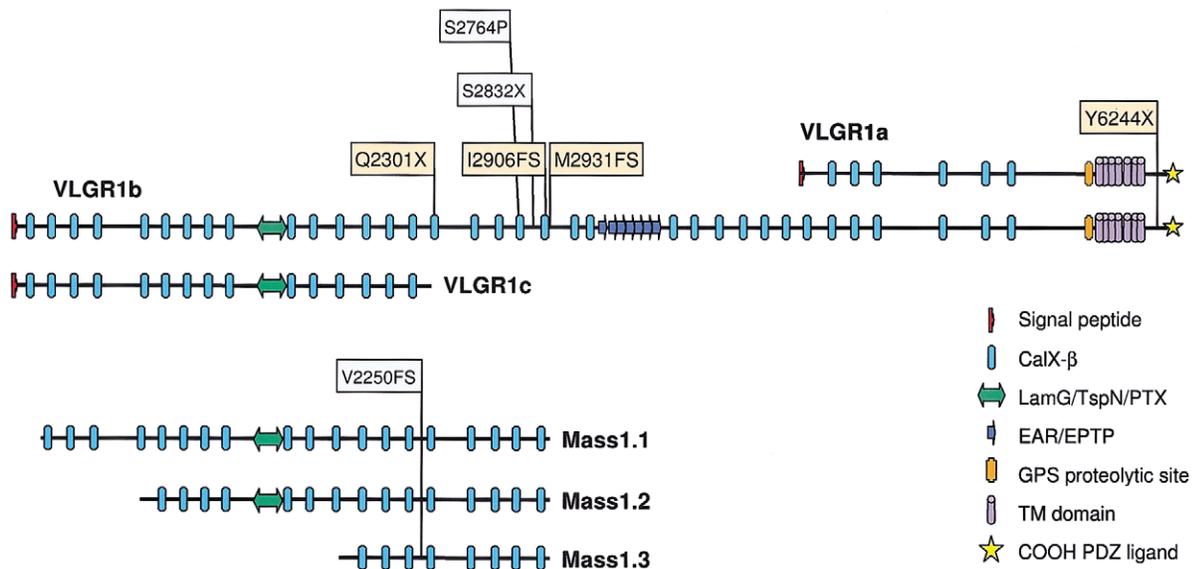
Because the USH2C phenotype manifests cochlear and retinal defects, we wanted to determine if any of the three *VLGR1* isoforms were tissue specific in these organs (fig. 3a and 3b). We performed semiquantitative RT-PCR by use of total RNA isolated from nine human fetal tissues. Primer pairs for the human isoforms *VLGR1a*, *VLGR1b*, and *VLGR1c* have been described elsewhere (McMillan et al. 2002). Total RNA from three human tissues (fetal brain, fetal liver, and placenta) was purchased from Clontech. For cDNA synthesis, total RNA was treated with DNase I (Invitrogen), according to the manufacturer's conditions, and random-primed in a volume of 250  $\mu$ l from 3.1  $\mu$ g total RNA using 4 pmol random hexanucleotides, 10 mM Tris-HCl, pH

8.3, 50 mM KCl, 0.01% (v/w) gelatin, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 95 U RNAGuard (Pharmacia), and 2,500 U MMLV reverse transcriptase (Invitrogen). Semiquantitative RT-PCRs were performed by sampling 50  $\mu$ l RT-PCR reactions at the 25th, 30th, 35th, and 40th cycle. PCR reactions included 87 ng of randomly primed cDNA, 11.2 pmol of each primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% gelatin, 2.5–3 mM MgCl<sub>2</sub>, 160 mM of each dNTP, and 1.75 U *Taq* DNA polymerase (Invitrogen) under the following conditions: 92°C for 4 min, followed by 40 cycles of 95°C for 30 s, 50°C / 55°C for 30 s, and 72°C for 1 min, with a final step at 72°C for 10 min. RT-PCR results show expression of all three *VLGR1* isoforms from fetal retina and cochlea, confirming the EST expression profile of *VLGR1*. High relative expression in fetal brain and spinal cord confirms the neuronal expression of *VLGR1* (McMillan et al. 2002) (fig. 3a and 3b). Although there appears to be a consistent difference in the amount of *VLGR1a*, *VLGR1b*, and *VLGR1c* RT-PCR products amplified from different tissues, no apparent tissue-specific differences in the relative abundance between the three isoforms was seen in any of the fetal tissues tested (fig. 3a and 3b). In addition, we find the relative amount of the *VLGR1a* and *VLGR1b* isoforms to be roughly equal as judged by RT-PCR, which is in contrast to an approximately fourfold difference in expression of the *VLGR1b* isoform over

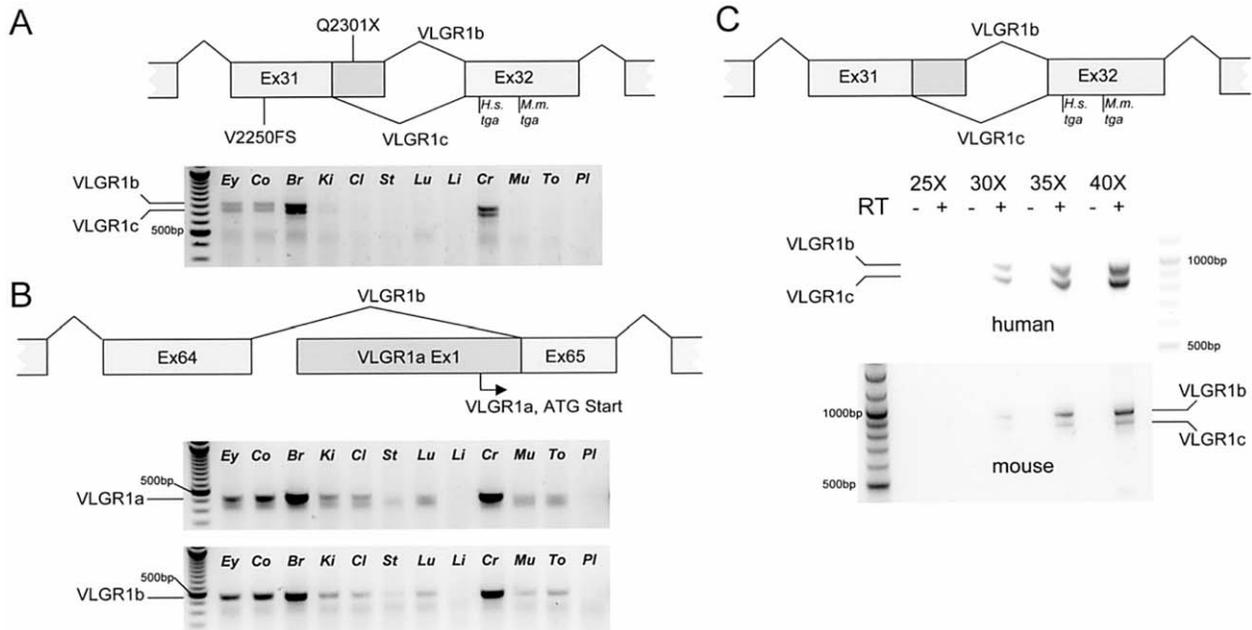
the VLGR1a isoform previously observed in human fetal kidney, brain, and lung (McMillan et al. 2002). One explanation for this disparity could be the way the RT-PCR of the VLGR1a and VLGR1b isoforms was performed, as the study by McMillan et al. (2002) amplified VLGR1a and VLGR1b in multiplex, whereas we chose to amplify them in separate reactions because of a significant difference in the TMs of the sense primers.

The VLGR1b and VLGR1c isoforms differ by 83 bp as a result of the alternative exon 31 donor splicing (Skradski et al. 2001; McMillan et al. 2002). A mouse *Vlgr1* mutation in exon 31 (7009delG; V2250FS) is responsible for the monogenic audiogenic seizure susceptible (MASS) phenotype (Skradski et al. 2001). The *Vlgr1*<sup>7009delG</sup> (*Vlgr1*<sup>Mass1</sup>) mouse allele mutates both mouse *Vlgr1b* and *Vlgr1c* splice isoforms, whereas the human exon 31 *VLGR1* mutation (Q2301X), in addition to the other three *USH2C* mutations, affects VLGR1b (figs. 2 and 3). To determine if species-specific differences in the VLGR1b and VLGR1c isoforms might help to explain the *VLGR1* mutant phenotypes, RT-PCR of VLGR1b and VLGR1c isoforms from mouse and human fetal RNA was performed. For RT-PCR of the VLGR1b and VLGR1c isoforms, random-primed total mRNA from human and mouse fetal brain RNA (Clontech) was reverse transcribed with Superscript III (Invitrogen), according to

standard conditions, and PCR with AmpliTaq Gold (PE Biosystems) was as described for genomic PCR amplification, except that the amount of MgCl<sub>2</sub> was 3.0 mM. Amplification primers for the mouse *Vlgr1b* and *Vlgr1c* isoforms were as follows: sense 5'-CGAACTGGAGGAACATTTGCAG-3' and antisense 5'-GCAGACACGTT CACCCAAGTTC-3'. Cloning and sequencing of VLGR1b and VLGR1c RT-PCR products from both human and mouse confirm the evolutionary conservation of the alternative exon 31 splice donor sites in both species (data not shown). It is not surprising that, although the VLGR1b messages of human and mouse share 81% sequence identity overall, the last 113 bp of exon 31 are 100% conserved at the DNA level, indicating that the sequence context of the mRNA in this region is critically important, perhaps defining a critical *cis* element necessary for alternative mRNA processing. A comparison of RT-PCR products from fetal human and mouse brain revealed a difference in the relative abundance of VLGR1b and VLGR1c isoforms, indicating a species-specific difference in mRNA processing through the alternative exon 31 donor sites (fig. 3c). These results suggest that isoform-specific mutations in *VLGR1*, either alone or in combination with a specific difference in mRNA processing, may underlie the manifestation of phenotype in these species.



**Figure 2** Domain structure of VLGR1/MASS1 isoforms. Conceptual translation of VLGR1b mRNA reveals a protein with a large ectodomain with 35 Calx- $\beta$  domains, 1 LamG/TspN/PTX homology domain (Beckmann et al. 1998), 7 EAR/EPTP repeats forming a putative  $\beta$ -propeller folding domain (Scheel et al. 2002; Staub et al. 2002), a GPS, a B-family 7TM domain, a putative intracellular tail with multiple potential serine phosphorylation sites, and a putative PDZ-binding COOH-terminal end (McMillan et al. 2002). The PTX domains of VLGR1 and the *USH2A* protein usherin are most similar to one another, with a BLASTP score of  $2e-09$ . The locations of human *USH2C* (yellow) and febrile-seizure (FS; gray)-associated mutations are shown on VLGR1a, VLGR1b, and VLGR1c isoforms. The mouse audiogenic reflex seizure model *Frings* and the age-related hearing loss model BUB/BnJ are both homozygous for the V2250FS mutation and are noted on Mass1.1, Mass1.2, and Mass1.3 isoforms (Skradski et al. 2001).



**Figure 3** RT-PCR of VLGR1 mRNA splice forms from human and mouse fetal tissue. Human fetal tissues from left to right: eye (Ey), cochlea (Co), brain (Br), kidney (Ki), colon (Cl), stomach (St), lung (Lu), liver (Li), spinal cord (Cr), muscle (Mu), tongue (To), and adult placenta (Pl). **A**, The relative locations of the human Q2301X and the *Frings* and BUB/BnJ mouse V2250FS mutations to the alternative donor site that differentiates the VLGR1b and VLGR1c splice isoforms. These isoforms differ by 83 bp because of the alternative use of a 5' splice-donor site in exon 31 (Skradski et al. 2001; McMillan et al. 2002). Expression from eye and cochlear tissue is less intense at 30 cycles of RT-PCR than from brain and spinal cord. At 40 cycles, all tissues but liver and placenta show VLGR1 expression of similar relative intensities for VLGR1c, compared with VLGR1b (fig. 2b; data not shown). **B**, The location of the VLGR1a 5' start site, relative to the exon 65 3' splice acceptor of VLGR1b. RT-PCR results at 30 cycles for VLGR1a and VLGR1b splice forms show a similar tissue distribution to that in figure 2a, as well as similar relative intensities. All RNAs were positive for D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (data not shown). **C**, A comparison of VLGR1b and VLGR1c concentration in human and mouse reveals a species-specific difference in the relative abundance of these isoforms, which were amplified from fetal brain mRNA, indicating a difference in the use of the alternate exon 31 donor sites between species.

In situ hybridization of mouse embryo sections had shown *Vlgr1* expression concentrated in the ventricular zone where neural progenitor cells reside during embryonic neurogenesis, suggesting that the *Vlgr1b* and *Vlgr1c* isoforms play a role in the development of the murine CNS (McMillan et al. 2002). This idea is strengthened by the association of the MASS phenotype with the *Vlgr1* mutant allele (Skradski et al. 2001). The *Vlgr1* mouse ortholog includes in its open reading frame three additional and distinct mRNAs previously characterized as isoforms of the mouse *Mass1* gene (*Mass1.1*, *Mass1.2*, and *Mass1.3*) on mouse chromosome 13 (Skradski et al. 2001) (fig. 2). The *Frings* mouse has not been reported to have deficient hearing or vision; however, no histological or electrophysiological data have been published that explicitly rule out such possibilities. In addition to *Frings*, the BUB/BnJ inbred mouse was also found to be a *Vlgr1<sup>Mass1</sup>* homozygote, and it exhibits the MASS phenotype until a progressive age-related hearing loss (AHL) phenotype manifests in this strain by 3 wk of age (Skradski et al. 2001). BUB/BnJ is one

of several inbred mouse strains exhibiting AHL (Zheng et al. 1999). The variable severity and progression of hearing loss in many AHL mouse strains, including BUB/BnJ, is influenced by a major modifier locus on mouse chromosome 10 (*ahl*, also the modifier-of-deaf-waddler locus, *mdfw*). Both *ahl* and *mdfw* are caused by the *Cdh23<sup>753G→A</sup>* splicing mutation, a synonymous transition of the last nucleotide of exon 7 (Noben-Trauth et al. 2003). It remains to be determined if the early onset of hearing loss in BUB/BnJ is influenced by the *Vlgr1<sup>Mass1</sup>* mutation, as has been shown for other AHL "accelerating alleles" in combination with *Cdh23<sup>753A</sup>* (Noben-Trauth et al. 2003). It should be noted that BUB/BnJ is also homozygous for *rd1*, a mutation in the *Pde6b* gene that results in early photoreceptor-cell apoptosis because of a lack of cGMP phosphodiesterase activity. The fact that BUB/BnJ carries homozygous mutations in *Pde6b* (*rd1*), *Cdh23* (*ahl*, *mdfw*), and *Vlgr1* (*Mass1*) suggests caution should be used in interpreting the phenotypic effects of these genes in this mouse strain.

The VLGR1 protein belongs to a 33-member sub-

group of the large N-terminal family B (LNB) seven-transmembrane (7TM) receptors. Of the 33 LNB 7TM members, 32 are orphan receptors and only a few have demonstrated specific G-protein signaling (Foord et al. 2002). All LNB 7TM members, including VLGR1, have a G-protein-coupled proteolysis site (GPS); cleavage at this site is thought to be a requirement for the proper expression of this class of 7TM receptors on the cell surface (Krasnoperov et al. 2002). VLGR1 may function, like other LNB 7TM members, as a natural protein chimera, with an extracellular cell-adhesion subunit and a canonical G-protein-coupled receptor subunit that are linked by noncovalent interactions (Krasnoperov et al. 2002). The possible function of VLGR1 might be best explored by trying to predict the functions of the two putative subunits separately.

The C-terminal residues of VLGR1 correspond to the consensus motif that is recognized as a ligand for the class I subfamily of PDZ domains (PDZ-binding interface [PBI] [Nikkila et al. 2000; Hung and Sheng 2002]). Interestingly, the C-termini of cadherin 23, protocadherin 15, harmonin, and SANS, all proteins with mutations that cause the USH1 phenotype, have class I PBIs. An interaction through the 2nd PDZ domain of harmonin and the C-terminal PBI ligand of cadherin 23 has been independently confirmed (Boeda et al. 2002; Siemens et al. 2002). Colocalization of harmonin and SANS in transfected HeLa cells suggests that these proteins also interact, perhaps through a PDZ-PBI interaction (Weil et al. 2003). The fact that all USH1 protein mouse mutants, including the nonmuscle myosin VIIa, which also has a protein interaction with harmonin, phenotypically present with defects in hair cell stereocilia development suggests that these proteins function as a macromolecular complex necessary to shape and maintain the stereocilia as an organized cohesive unit (Boeda et al. 2002; Siemens et al. 2002; Weil et al. 2003). It remains to be determined whether one or more of the three PDZ domains of harmonin—or other PDZ domain-bearing protein—have a high-affinity interaction with the PBI of VLGR1.

The very large extracellular portion of VLGR1b has a total of 35 CalX- $\beta$  modules (fig. 2), named for the homology shared between the regulatory domains of Na<sup>+</sup>/Ca<sup>2+</sup> exchanger proteins and the cytoplasmic portion of integrin- $\beta$ 4 (Schwarz and Benzer 1997; McMillan et al. 2002). Portions of the extracellular domain of VLGR1a that include a subset of the CalX- $\beta$  domains bind Ca<sup>2+</sup> and other cations (Nikkila et al. 2000). Because of the extensive CalX- $\beta$  modules, one might postulate that the molecule acts as an extracellular Ca<sup>2+</sup> sink, as an extracellular Ca<sup>2+</sup> monitor sensitive to the regulation of intra- and extracellular Ca<sup>2+</sup> trafficking, or in Ca<sup>2+</sup>-dependent cell adhesion. Disruption of normal Ca<sup>2+</sup> metabolism is known to affect both hearing

and vision. For example, mutations in the plasma membrane Ca<sup>2+</sup> ATPase pump Atp2b2 (PMCA2) cause the deaf waddler (*dfw*) phenotype in mice (Kozel et al. 1998; Street et al. 1998). It has been postulated that the murine *abl* gene (*mdfw*, *Cdh23*<sup>753A</sup>) acts epistatically to cause an age-dependent hearing loss through an interaction between the Ca<sup>2+</sup>-extruding PMCA2 channel and the calcium-dependent homotypic cell-adhesion properties of the cadherin 23 protein (Noben-Trauth et al. 1997, 2003). Furthermore, a functional interaction has been postulated between PMCA2 and the sodium bicarbonate cotransporter, NBC3, at the photoreceptor synapse, as the loss of H<sup>+</sup> buffering by NBC3 causes retinal dystrophy (and hearing loss) in NBC3 knock-out mice (Bok et al. 2003). The possibility that calcium homeostasis is involved in USH2C is especially intriguing in light of the observation that the Ca<sup>2+</sup>-channel-blocking drug, diltiazem, is protective against noise-induced hearing loss (Maurer et al. 1998; Heinrich et al. 1999). However, there is conflicting evidence about whether diltiazem is neuroprotective for the retina, since none of the animal models tested had RP resulting from a calcium metabolism defect (Frasson et al. 1999; Pasantes-Morales et al. 2002; Pawlyk et al. 2002).

The large VLGR1b ectodomain has two recognized potential protein-interaction domains, LamG/TspN/PTX (Beckmann et al. 1998) and EAR/EPTP (Scheel et al. 2002; Staub et al. 2002) (fig. 2). It has been suggested that the multiple Ca<sup>2+</sup>-binding CalX- $\beta$  modules may mediate the protein-protein interactions of these two domains (McMillan et al. 2002). The EAR/EPTP motif occurs as a repeated set of domains that together are postulated to form a seven-bladed-propeller structure that, in other proteins, acts as a highly specific receptor (Pons et al. 2003). Probable ligands for the EAR/EPTP domain of VLGR1 would be extracellular or cell-adhesion proteins. Usherin, the product of the *USH2A* gene, is a known extracellular basement-membrane protein (Bhattacharya et al. 2002). When usherin is defective, the phenotype is remarkably similar to that seen in patients with inactivated VLGR1. Immunolocalization of usherin to the perinurium of the spiral ganglion cells of the cochlea (Bhattacharya et al. 2002) and the likely neuronal localization of the VLGR1 receptor indicates that VLGR1 and usherin may colocalize in specific subcellular compartments. Furthermore, the significant homology between the LamG/TspN/PTX motifs of VLGR1 (fig. 2) and usherin indicates that the two proteins may share an affinity for a common binding partner, reinforcing the hypothesis of an interaction between these proteins. Whether usherin is the extracellular ligand or another yet-to-be-identified USH2 protein remains to be established.

Because *VLGR1* is expressed as at least three different isoforms in the human, questions arise as to which of

the isoforms is responsible for USH2C and, as a corollary, whether mutations involving other isoforms cause a different phenotype, especially a seizure disorder. The USH2C mutations we have observed all involve isoform VLGR1b, but not isoform VLGR1c. The *Frings* mutation affects both VLGR1b and VLGR1c. We specifically asked all of the patients with USH2C in our study about seizures of any type and received uniformly negative responses. It is plausible to hypothesize that the *Frings* seizure disorder is primarily due to mutations affecting the VLGR1c isoform. Whether a functional protein is encoded by VLGR1c will be important in helping to determine whether the disparity in phenotype between human and mouse *VLGR1* mutants are governed by species-specific differences in VLGR1 function, the inactivation of a subset of VLGR1 isoforms, or a combination of the two. The fact that the *VLGR1* S2832X and S2764P mutations (isoform VLGR1b) segregate in families with both febrile and afebrile seizures (FEB4 [MIM 604352]) suggests a causal relationship between human seizures and *VLGR1*. But the relative low frequency of FEB4-associated mutations, the lack of a significant association between *VLGR1* SNPs and FEB4, and the lack of any self-reported seizure-related phenotype in our USH2C patients suggest that VLGR1b is not a significant contributor to any seizure disorder (Nakayama et al. 2002).

All 13 patients with USH2C and *VLGR1* mutations are female, a highly significant deviation from expectation ( $P < .002$ , Fisher's exact test). The biologic significance, if any, of this observation can only be speculated on. Although some selection bias could conceivably play a role, it is difficult to understand why the shift in sex ratio is so extreme. A likely explanation is that males are being excluded from the sample: the male phenotype might be more severe, possibly lethal; it could be associated with some other hearing-/vision-loss syndrome; or the phenotype could be very mild or nonexistent. Nonetheless, the possibility that an endocrine-based component could influence the Usher syndrome phenotype as a modifier of *VLGR1* expression and/or protein function is intriguing. Mutations in *VLGR1* do not seem to be responsible for a large proportion of the USH2 cases in our sample, despite the fact that the gene is large and one might have expected otherwise. The possibility of male lethality could explain the apparently low contribution of *USH2C* mutations to the total USH2 mutational load.

The discovery of USH2C-associated *VLGR1* mutations poses several interesting and important questions regarding the etiology of USH2. Localization and functional studies, in addition to thorough genetic screening for additional *VLGR1* mutations, will be needed to elucidate the role of VLGR1 in the development, maintenance, and function of vision and hearing. Furthermore, its capacity as a putative G-coupled receptor makes it

a legitimate target in the search for possible therapeutics that may have a broad impact on all types of Usher syndrome.

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## Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

National Eye Institute Gene Bank, <http://neibank.nei.nih.gov/libs/NbLib0013/NbLib0013.shtml> (for human retina) and <http://neibank.nei.nih.gov/libs/NbLib0011/NbLib0011.shtml> (for human fetal cochlea)  
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for USH2, USH1D, USH1F, USH2C, and FEB4)  
 UCSC Genome Browser, <http://genome.cse.ucsc.edu/> (for human *VLGR1*, AF435925; mouse *Vlgr1*, AF435926; and *VLGR1* genomic DNA, NT\_028179)

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