Iron metabolism mutant *hbd* mice have a deletion in *Sec15l1*, which has homology to a yeast gene for vesicle docking

Robert A. White a,b,*, Leigh A. Boydsto n a, Terri R. Brookshier a, Steven G. McNulty a, Ndona N. Nsumu a, Brandon P. Brewer a, Krista Blackmore a

a Department of Medical Research, Children’s Mercy Hospitals and Clinics, Kansas City, MO 64108, USA
b Department of Pediatrics–Genetics, University of Missouri at Kansas City School of Medicine, Kansas City, MO 64108, USA

Received 11 July 2005; accepted 22 September 2005
Available online 11 November 2005

Abstract

Defects in iron absorption and utilization lead to iron deficiency and anemia. While iron transport by transferrin receptor-mediated endocytosis is well understood, it is not completely clear how iron is transported from the endosome to the mitochondria where heme is synthesized. We undertook a positional cloning project to identify the causative mutation for the hemoglobin-deficit (*hbd*) mouse mutant, which suffers from a microcytic, hypochromic anemia apparently due to defective iron transport in the endocytosis cycle. As shown by previous studies, reticulocyte iron accumulation in homozygous *hbd/hbd* mice is deficient despite normal binding of transferrin to its receptor and normal transferrin uptake in the cell. We have identified a strong candidate gene for *hbd*, *Sec15l1*, a homologue to yeast SEC15, which encodes a key protein in vesicle docking. The *hbd* mice have an exon deletion in *Sec15l1*, which is the first known mutation of a SEC gene homologue in mammals.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Iron metabolism; Mouse mutant; Anemia

Iron is fundamental to the biology of living organisms and, until recently, iron transport remained poorly understood [1,2]. Critical genes responsible for iron homeostasis have been identified by positional cloning with the microcytic anemia (*mk*) and the sex-linked anemia (*sla*) mutant mice [3,4], in addition to the study of iron metabolism defects in human and zebrafish [5–9]. However, other genes playing essential roles in iron homeostasis have yet to be discovered. This includes the genes responsible for transferring iron from the endosome to the mitochondria for the production of heme.

Diferric transferrin binds to the transferrin receptor (TfR) on the cell membrane, and the transferrin–TfR complex is internalized into clathrin-coated vesicles by endocytosis [10]. The vesicles lose their clathrin coat and fuse with endosomes, in which acidification occurs, and iron is released from transferrin. DMT1 (divalent metal transporter-1) protein facilitates the passage of iron through the endosomal membrane into the cytoplasm [3]. Recently, an essential transporter for iron importation into the mitochondria was discovered and is disrupted in zebrafish *frascati* mutants with profound hypochromic anemia. The *frascati* gene product is a novel member of a mitochondrial solute transporter family that transports metabolites across the inner mitochondrial membrane [11]. However, it is still unclear how iron is transported from the endosome to the mitochondria. It has been proposed that the highly efficient transfer of iron from endosome to mitochondria in normal reticulocytes may result from the endosome navigating the cell to dock onto the mitochondria to transfer iron directly to the latter (the so-called “kiss and run” hypothesis) [12].

The hemoglobin-deficit mouse mutant (gene symbol *hbd*) exhibits a severe, microcytic anemia, which is not due to iron deficiency since serum iron levels are above normal [13]. Hemoglobin levels are reduced in *hbd* mice and zinc protoporphyrin levels are increased, indicating a deficiency in heme synthesis. Previous studies have shown that the *hbd* mouse has a defect in iron utilization, presumably in the release of iron from the endosome in reticulocytes [12–14]. Although transferrin uptake was normal in reticulocytes from *hbd/hbd* and +/+ mice, iron acquisition was significantly reduced in
hbd/hbd reticulocytes [14]. Diferric transferrin appears to bind to the transferrin receptor in the hbd reticulocytes and enters the cells by the endosomal pathway. However, iron does not accumulate in the cells. Therefore, a defect occurs in iron acquisition distal to the binding of transferrin to its receptor. A comparable mutant has been reported in a Chinese hamster ovary cell line that has a defect in transferrin-receptor-mediated endocytosis [15]. Binding and internalization of transferrin proceed normally in this hamster cell line but iron does not accumulate in the cell, similar to the case for hbd reticulocytes.

The phenotype of hbd mice can be transferred by bone marrow transplantation to irradiated mice, showing that the defect is intrinsic to the bone marrow [16]. Conversely, hbd mice can be cured by transplantation of normal bone marrow [17] but are not cured by intraperitoneal iron–dextran injections. Collectively, these data suggest that iron utilization in the bone marrow is the primary defect.

The hbd mouse arose as a spontaneous mutation and is inherited in an autosomal recessive manner [13]. The hbd gene has been mapped to mouse chromosome 19, but the gene has not been previously identified [18]. We set out to positionally clone the hbd mutant gene and now report the identification of a strong candidate gene for hbd, Sec15l1, a homologue to the yeast gene encoding vesicle docking protein, SEC15 [19–23].

Results

Hematology of hbd mice

Blood samples were obtained from adult C57BL/6J hbd/hbd, +/+hbd, and +/+ mice. Table 1 illustrates marked disturbances of red blood cell parameters in hbd/hbd mice. Homozygous hbd/hbd mice showed a significant reduction (p < 0.05) in hematocrit and hemoglobin values. The mean cell volume (MCV) shows that the anemia in hbd mice is a microcytic anemia. Also, there was a significant elevation in Zn protoporphyrin levels in hbd mice, suggesting ineffective utilization of iron and deficient hemoglobin production.

High-resolution mapping of the hbd locus

A low-resolution backcross study was previously used to locate hbd on chromosome 19 in mouse [18]. We set out to positionally clone the hbd gene using a high-resolution map of the mating of B6MOLF1 +/+hbd with C57BL/6J hbd/hbd mice to generate 2454 backcross mice. The MOLD/Rk strain is highly polymorphic and allowed the identification of the greatest number of genetic markers for the mapping of the hbd locus. The backcross mice were scored by measuring the level of Zn protoporphyrin, which is elevated in hbd anemic mice, from peripheral blood samples. Using eight dinucleotide CA-repeat microsatellite genetic markers, we were able to define a critical region for the hbd gene. Of 2454 backcross mice we found no recombinant types for one marker (D19Mit101) (Fig. 1). The critical region (in which the hbd gene must be located) was defined by the flanking markers Kif11, at 0.24 cM on the proximal side (with six crossover backcross mice), and Fer1l3, at 0.04 cM on the distal side (with one crossover backcross mouse) (Fig. 1). The calculated size of the hbd critical region, which includes all of these genetic markers, is 836 kb as determined by the sequence available in GenBank contig NT 039689. It was estimated that Fer1l3 was only 80 kb from the hbd locus based on the average length of genomic DNA per centimorgan (2000 kb/cM) across the mouse genome. GenBank contig NT 039689 contains three functional genes in this region: Hhex (hematopoietically expressed homeobox), Sec15l1 (secretion protein 15-like 1), and Cyp26a1 (cytochrome P450, family 26, subfamily a1). Haplotype data for the backcrosses are shown in Fig. 1.

Mutation analysis of the hbd gene

Our approach for mutation analysis was to amplify exons for each gene in the hbd critical region by genomic PCR using primers derived from flanking sequence. The results were examined for gene deletions, insertions, or large rearrangements as determined by the size of the PCR product generated from normal and mutant DNA. The only gene in the hbd critical region with a defect was the Sec15l1 gene, which was shown to have a deletion of exon 8 in hbd genomic DNA (Fig. 2a). All other functional genes in the critical region were amplified and sequenced for control C57BL/6J and hbd mice. None showed large deletions, insertions, or rearrangements and there were no sequence mutations except for a silent polymorphism in exon 2 of the Hhex gene, which also was present in multiple mouse strains (SWR/J, AKR/J, CAST/Rk, A/J, Balbc/J, and WB/Re). Sec15l1 exons in C57BL/6J and hbd mice were also sequenced to confirm similarity to the Sec15l1 GenBank sequence. Several backcross progeny were randomly selected to verify the deletion of exon 8 in Sec15l1 in hbd mice by genomic PCR (Fig. 2b). In addition, exon 8 of Sec15l1 was amplified from the genomic DNA of 12 mouse strains (A/J, AKR/J, Balbc/cJ, C57BL/10J, CAST/Rk, C57L/6J, C57L/6J, DBA/2J, MOLD/Rk, SWR/J, WB/Re, and 129svJ) to show that the absence of exon 8 in hbd mice was not a polymorphic form of the gene (data not shown).

The Sec15l1 exon 8 deletion in hbd mice was verified by long-range genomic PCR. Using primers derived from exon 7 and exon 9, long-range genomic PCR of hbd and wild-type DNA revealed a 4.6-kb downward shift from 7.6 to 3.0 kb, consistent with the Sec15l1 gene deletion (Fig. 3a). Genomic PCR, followed by DNA sequencing, was also used to
determine the exact site of the deletion of Sec15l1 in hbd mice. A 1.0-kb genomic PCR amplicon, which spanned the Sec15l1 deletion, was generated by using primers within intron 7 and intron 8. The hbd deletion extends from nt 28,707 in intron 7 to nt 33,247 in intron 8 of Sec15l1 (GenBank contig NT 039689). The deleted DNA sequence in hbd mice is replaced by 38 bp of DNA starting at nt 28,707 of the Sec15l1 gene and one recombinant for the Kif11 locus and one recombinant for Fer1l3. CA repeat polymorphisms for the backcross were found for the following genes starting at the nucleotide number listed: Kif11 nt 1328, Fer1l3 nt 40,858, Rbp4 nt 5332, and Cpeh3 nt 148,241. Genetic markers can be found at www.informatics.jax.org and in GenBank, Accession No. NT 039689.

Expression of the hbd mutation

Expression of the Sec15l1 gene in normal +/+, hbd, and heterozygous +/hbd mice was examined by RT-PCR using primers whose products extend from Sec15l1 exon 7 to exon 9. Consistent with an exon 8 deletion, the RT-PCR product from hbd/hbd mice showed a smaller Sec15l1 transcript (157 bp) than the amplicon from normal reticulocyte RNA (226 bp) (Fig. 5a). The +/hbd RT-PCR sample showed both bands (226 and 157 bp) as expected for heterozygosity of the deletion mutation. As a result of the exon 8 deletion, Sec15l1 expression was predicted to result in an in-frame transcript spliced from exon 7 to exon 9. To verify the in-frame deletion, the 157-bp Sec15l1 amplicon from the hbd reticulocyte RT-PCR was isolated and sequenced (Fig. 5b). The resulting nucleotide sequence confirms that exon 7 is spliced onto exon 9 and the coding sequence is in-frame. Exon 8 of the Sec15l1 gene is 69 bp in length and codes for exactly 23 amino acids. Consequently, a truncated SEC15L1 protein (90 kDa) is predicted to be produced in hbd mice compared to full-length SEC15L1 protein in normal mice (93 kDa). Among the amino acids deleted in the hbd SEC15L1 protein, there are 2 amino acids important for tertiary structure: a cysteine residue at position 283 and a proline residue at position 287.

Normal expression of Sec15l1 was examined in wild-type mice by RT-PCR using a forward primer for exon 7 and reverse primer for exon 9. Sec15l1 RNA was detected in multiple tissues, including reticulocyte, spleen, bone marrow, kidney, brain, liver (Fig. 5c), and duodenum (Fig. 5d). Therefore, Sec15l1 RNA is expressed in nonhematopoietic tissues (kidney, brain, and duodenum) in addition to hematopoietic tissues (reticulocyte, spleen, bone marrow, and liver).

Discussion

The products of at least 14 genes, including SEC15, are involved specifically in vesicular transport from the Golgi apparatus in the yeast Saccharomyces cerevisiae [22]. The exocyst complex is specifically located at the site of vesicle fusion [19]. The mouse SEC15L1 protein is similar to SEC15 of yeast, but its specific function has not been determined. Sec15l1 may be involved in vesicle docking like its yeast homolog. The peptide homology of mouse SEC15L1 to yeast SEC15 is 19%, whereas the peptide homology between human and mouse SEC15L1 is 92%. There are at least 26 SEC-like genes found in the mouse genome (www.informatics.jax.org), and this is the first known mutation of a SEC-like gene in mammals.

The key to understanding the defect in hbd mice presumably lies in its involvement in the endocytic pathway in which endosomes are formed, iron is released, and the endosomes are

Fig. 1. High-resolution genetic map of the hbd critical region on mouse chromosome 19. Relative distances in centimorgans (cM) are shown. The segregation pattern for the phenotype and genetic markers is indicated on the right. The black squares represent the +/hbd heterozygous genotypes, while the white squares represent the homozygous hbd/hbd phenotypes. The numbers of mice with the haplotypes are indicated below. The asterisks refer to the animals used to define the critical region for the hbd locus with six recombinants for the Kif11 locus and one recombinant for Fer1l3. CA repeat polymorphisms for the backcross were found for the following genes starting at the nucleotide number listed: Kif11 nt 1328, Fer1l3 nt 40,858, Rbp4 nt 5332, and Cpeh3 nt 148,241. Genetic markers can be found at www.informatics.jax.org and in GenBank, Accession No. NT 039689.

Fig. 2. Mutation analysis of the Sec15l1 gene in hbd mice. (a) Genomic PCR of exons 1 through 22 of the Sec15l1 gene in control C57BL/6J +/+ and C57BL/6J hbd/hbd DNA shows deletion of exon 8 in the hbd mouse (indicated by asterisk). Molecular weight marker (M) is a 100 bp ladder. (b) Randomly selected backcross mouse DNAs (+/hbd normal, N, and hbd/hbd) were amplified for exon 8 of the Sec15l1 gene. Concordance of the deletion with the hbd phenotype is perfect.
Sec15l1, having homology to a gene encoding a vesicle docking protein, appears to be an excellent candidate gene for hbd based on the finding that the endocytosis cycle seems to be affected in hbd reticulocytes [14]. A mutation in the Sec15l1 gene is consistent with the hbd mutant phenotype since TfR-mediated endocytosis involves vesicle docking.

There are two possibilities for the effect of the Sec15l1 mutation on iron metabolism in hbd reticulocytes. First, the absence of normal SEC15L1 protein in hbd reticulocytes may have an effect on iron transport by not allowing the docking of the iron-containing vesicles to the endosome. Iron-containing vesicles would likely accumulate in hbd reticulocytes under these conditions and, in this case, iron would be expected to increase in the mutant cells. However, previous studies have shown that iron accumulation is lower than normal in hbd reticulocytes [14] and therefore, this type of docking defect may not be the primary defect. An alternative hypothesis is that SEC15L1 may be a key protein in iron targeting to the mitochondria. A previous proposal suggests that there is a
migration of endosomes through the cell to mitochondria and, once there, the endosomes dock onto the mitochondria to release iron directly to the organelle [12]. Furthermore, iron is transported out of the endosome by DMT1 to protein(s) on the mitochondrial membrane. The Sec15l1 mutation in hbd mice may interfere with the docking of endosomes to mitochondria. In the case of hbd reticulocytes, iron may not be transferred efficiently from the endosome to the mitochondria because vesicle docking is impaired. Absent or abnormal Sec15l1 protein in hbd mice may prevent the transient association of the endosome with mitochondria and, therefore, not allow the transport of iron from one organelle to the other. The hbd endosome may cycle back to the cell surface without transferring iron to mitochondria and instead release iron outside the cell.

The hbd mutant suffers a hypochromic, microcytic anemia but has no other phenotypic defects. This suggests that Sec15l1 must have a specific function since no tissues other than erythroid cells appear to be affected. The apparent lack of compensation by any of the other 26 mouse SEC-like genes to overcome the mutant phenotype in the hbd mouse also suggests that other SEC-like gene products have their own specific function independent of Sec15l1. In addition, it is not surprising that the Sec15l1 mutation appears to affect only iron transport in hbd mice since there is evidence that mutations in one endocytic pathway do not affect other endocytic pathways. For example, a familial hypercholesterolemia mutation in the LDL receptor-mediated endocytosis pathway does not affect the transferrin-mediated iron transport pathway [24]. Therefore, Sec15l1 is likely to play a specific role in one pathway alone.

Sec15l1 RNA is expressed in hematopoietic tissues as determined by RT-PCR (which is expected for a protein with a role in iron utilization for the production of heme). However, Sec15l1 RNA is also detected in nonhematopoietic tissues. This is not surprising since even the major iron transporter DMT1 is expressed in many diverse nonhematopoietic tissues in addition to hematopoietic tissues. The deletion of exon 8 in the Sec15l1 gene of hbd mice results in the flanking exons 7 and 9 being in-frame after splicing of Sec15l1 mRNA. The predicted outcome of the mutation is the production of a truncated protein (90 kDa) in hbd mice in contrast to the full-length Sec15l1 protein (93 kDa). The 23 deleted amino acids in the Sec15l1 protein of hbd mice include a cysteine and a proline, both of which are important to tertiary structure. There are multiple potential disulfide bridges made possible by several cysteine residues in the Sec15l1 protein and a deletion of one of these amino acids is likely to disrupt the normal tertiary structure of the protein. Therefore, the conformation of the mutant hbd Sec15l1 protein may be significantly changed and susceptible to proteolytic digestion. Immunological methods will be needed to verify whether the 90-kDa Sec15l1 truncated protein product is present in hbd reticulocytes or whether it is degraded. Ascertainment of the function of Sec15l1 can be established from protein–protein interaction studies, localization by immunocytochemistry, and the use of small inhibitory RNAs in murine erythroleukemia cells to determine the effect on iron absorption. Further analysis of normal Sec15l1 function will uncover its direct role in iron metabolism and whether it is involved in the mechanics of the final, crucial step of endosomal iron transport to mitochondria.

Materials and methods

Mice

C57BL/6J hbd/+ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The hbd mutants were identified by determining the levels of zinc protoporphyrin in peripheral blood. Mice with a value of zinc protoporphyrin >100 μg/dl were scored as anemic [18]. Peripheral blood samples were obtained from hbd/hbd and normal littermates. Zinc protoporphyrin was measured using a hematofluorometer Model 206D (AVIV Biomedical, Inc., Lakewood, NJ, USA). This instrument measures zinc protoporphyrin’s fluorescence and displays results in μg/dl.

Hematology studies

Peripheral blood samples were obtained in hematocrit tubes from adult C57BL/6J hbd/hbd, +/hbd, and +/+ mice. Red blood cells were counted with a Coulter counter (Model Z1; Miami, FL, USA). Hematocrit percentage was assessed using an Adams hematocrit reader (Becton–Dickinson, Parsippany, NJ, USA). The MCV was calculated from these values. Hemoglobin concentration of the red blood cells was assayed by Drabkin’s solution (Stanbio Laboratory, Boerne, TX, USA) was measured in a Perkin–Elmer Lambda 40 spectrophotometer (Norwalk, CT, USA) at a wavelength of 540 nm. Zinc protoporphyrin was measured using a hemato- fluorometer Model 206D (AVIV Biomedical, Inc.).

Genetic mapping

To generate a high-resolution map for the chromosomal region of the hbd mutation, C57BL/6J hbd/hbd mice were mated to the highly polymorphic MOLD/Rk strain. The (B6 × MOLD)F2 +/hbd progeny were backcrossed to the parental C57BL/6J hbd/hbd mice, which generated 2454 backcross offspring. The +/hbd normal and hbd/hbd anemic mice were scored, and high-molecular-weight DNA was extracted from spleens using the Super Quick Gene DNA extraction kit (Analytical Genetic Testing Center, Denver, CO, USA). These DNA samples were genotyped with primer pairs (Invitrogen, Carlsbad, CA, USA) as previously described [25] for a series of simple sequence length polymorphisms. Genotyping was also performed for four novel polymorphic CA repeats identified from four genes (Cpeh3, cytoplasmic polyadenylation element binding protein; Kif11, kinesin family member 11; Fer1l3, fer-like 3, also known as myoferlin; and Rbp4, retinol binding protein 4, plasma) from a contig sequence (GenBank Accession No. NT039689) of the hbd genomic region. The results of the mapping studies were analyzed using the Map Manager (v2.5) computer program (K. Manly, Roswell Park Institute, Buffalo, NY, USA) to determine the relative chromosomal position of the hbd locus.

Mutation analysis

Genomic PCR was performed using primers derived from flanking sequences 50 bp upstream and downstream of the exons in each gene from the hbd critical region. The primers were chosen using the Primer 3 program (frodo.wi.mit.edu/cgi-bin/prime3/prime3 www.cgi) from the sequence of a contig (GenBank Accession No. NT039689) that includes the hbd critical region. Oligonucleotide primers were prepared by Oligo Etc. (Wilsonville, OR, USA). Cycle conditions for genomic PCR were 1 min at 94°C, 1 min at 67°C, 1 min at 72°C for 35 cycles using the Fail Safe PCR kit (Epigen Technologies, Madison, WI, USA). Analytical gels were 3% GenePure 3:1 agarose (ICSB, Kaysville, UT, USA) using conditions specified by the manufacturer’s protocol for the Fail Safe PCR Kit. A 100 bp ladder (New England Biological Laboratories, Beverly, MA, USA) was used as a molecular weight marker. Genomic PCR for generating the large +/+ and hbd amplicons (7.6 and 3 kb) was performed using High Fidelity Platinum Taq DNA
polymerase (Invitrogen) according to the manufacturer’s protocol and run on a 1% GenePure 3:1 agarose (ISC prejudices) gel with a 1 kb ladder molecular weight marker (Invitrogen). PCR products were isolated using Micro Spin S-200 HR columns (Amersham Biosciences, Piscataway, NJ, USA) or CENTRI-SEP Spin Columns (Princeton Separations, Inc., Adelphia, NJ, USA) prior to sequencing. Amplicons were sequenced by the KU Medical Center Biotechnology Support Facility (Kansas City, KS, USA) using an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). Comparison of exon sequence was performed using the ClustalW multiple sequence alignment program (www.ebi.ac.uk/clustalw). Sequence data analysis was performed using the MacVector sequence analysis software (GGC, Madison, WI, USA). Primers were generated from the intron sequence flanking each Sec15I exon to prevent inadvertent amplification of other Sec15 family members. A Sec15I 226-bp cDNA probe was used for Southern blot analysis with techniques previously described [26]. The Sec15I cDNA probe was produced by PCR amplification of exons 7 to 9 from QUICK-Clone mouse kidney cDNA (BD Biosciences Clontech, Palo Alto, CA, USA). The probe was purified using Micro Spin S-200 HR columns (Amersham Biosciences).

RT-PCR analysis

RNA extractions from reticulocytes, spleen, bone marrow cells, kidney, brain, and liver were performed using the RNeasy Midi Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. RNA extracted from mouse duodenum was obtained from Zyagen Laboratories (San Diego, CA, USA). RT-PCR was completed using QuantiTect SYBR Green RT-PCR kit according to the manufacturer’s protocol. Analytical gels were the same as those described above for genomic PCR. Normal reticulocytes were obtained from phenylhydrazine-treated mice prepared as previously described [27]. This drug lyse red blood cells and induces reticulocytosis.

Acknowledgments

The authors thank Drs. M. Ray Brewer and Zohreh Talebizadeh for helpful discussion during the preparation of the manuscript.

References