Chromosomal Assignment of Genes Involved in Glycosylphosphatidylinositol Anchor Biosynthesis: Implications for the Pathogenesis of Paroxysmal Nocturnal Hemoglobinuria

By Russell E. Ware, Thad A. Howard, Tetsu Kamitani, Hui-Ming Chang, Edward T.H. Yeh, and Michael F. Seidin

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematologic disorder that affects both sexes equally. The biochemical defect in PNH resides in the incomplete enzymatic assembly of glycosylphosphatidylinositol (GPI) anchors used for surface protein attachment. In all PNH patients tested to date, the biosynthetic defect occurs at the addition of N-acetyl-glucosamine to the phosphatidylinositol molecule (class A defect). A human cDNA, Piga, that repairs cell lines with the class A GPI-anchor biosynthetic defect has been recently cloned. Mapping of Piga to the X chromosome suggests that a single acquired mutation within Piga could alter GPI-anchor synthesis and result in PNH. However, this finding does not explain why all PNH patients have the class A defect. In the current study, the chromosomal assignment of Piga, as well as of Pigf and Pigh, two additional genes involved in GPI-anchor biosynthesis, has been established using a mouse interspecific backcross mapping technique. In contrast to Piga, both human and mouse Pigf and Pigh genes map to autosomes. The location of Pigf and Pigh suggests that mutations on both alleles of these autosomal genes would be necessary to produce PNH. This helps to explain the predominant class A defect in PNH.

© 1994 by The American Society of Hematology.

MATERIALS AND METHODS

Mice. Two inbred strains of mice, C3H/HeJ-gld (designated CC) and Mus spreus (designated SS), were chosen as parent strains because they diverged evolutionarily several million years ago. As a result, unique restriction fragment length variants (RFLVs) exist for most of their genes. All mice were bred and maintained as previously described. M. spreus was chosen as the second parent in this cross because of the relative ease of detection of informative RFLVs in comparison with crosses using conventional inbred laboratory strains.

cDNA probes. The Piga cDNA probe was created using oligonucleotide polymerase chain reaction (PCR) primers derived from the published cDNA sequence. The 5' primer was GGACCATTCCCTTTTGGAT (nucleotides [nts] 541 to 560) and the 3' primer was GCCTTTCAACTCTTCACA (nts 1170 to 1151). These primers were used in a reverse transcriptase-PCR (RT-PCR) using total RNA.

From the Department of Pediatrics, Division of Hematology/Oncology; the Department of Medicine, Division of Rheumatology/Immunology, and the Department of Microbiology: Duke University Medical Center, Durham, NC; and the Department of Medicine and Anesthesiology, University of Texas Health Science Center, Houston, TX.

Supported by Grant No. ACS-IRG 158H (R.E.W.) from the American Cancer Society and Grants No. HL45851 (E.T.H.Y.) and HG-00734 (M.F.S.) from the National Institutes of Health. R.E.W. is the recipient of a James S. McDonnell Scholarship in Molecular Medicine, and E.T.H.Y. is an Established Investigator of the American Heart Association.

Address reprint requests to Russell E. Ware, MD, PhD, P.O. Box 2916, Division of Pediatric Hematology/Oncology, Duke University Medical Center, Durham, NC 27710.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.


3753

From www.bloodjournal.org by guest on November 16, 2017. For personal use only.
Gels. DNA was transferred to Nytran membranes (Schleicher and Schuell, Inc, Keene, NH), hybridized at 65°C with the cDNA probes, and then washed under stringent conditions, all as previously described. Clones used as probes in the current study (see Table 1) included the pPIGA5 (Piga), pPIGF1 (Pigf), and p11.15.7 (Pigh) clones derived from the HeLa cell line. The PCR product was subcloned using internal restriction endonuclease sites, and the actual Piga cDNA probe (pPIGA5) used for mapping spanned nts 590 to 1090.

The Pigf cDNA probe (pPIGF1) was also created using PCR primers derived from the published cDNA sequence. The 5' primer was GGGGATCCACATGAAAGATACGATAC (nts 3 to 18 with a 5' BamHI restriction site) and the 3' primer was GGGGTCGACATTTGTCTTGTAGTAAAGTG (nts 657 to 638 with a 5' SalI restriction site). RT-PCR using HeLa RNA was performed as above, followed by restriction endonuclease digestion and subcloning. The Pigh cDNA probe (p11.15.7) was isolated by complementation cloning of a human placental cDNA library into the class H mutant line Ltk-.15

Genotyping. DNA isolated from mouse organs by standard techniques was digested with various restriction endonucleases, and 10-μg samples of digested DNA were electrophoresed in 0.9% agarose gels. DNA was transferred to Nytran membranes (Schleicher & Schuell, Inc, Keene, NH), hybridized at 65°C with the cDNA probes, and washed under stringent conditions, all as previously described.16 Clones used as probes in the current study (see Table 1) included the pPIGA5 (Piga), pPIGF1 (Pigf), and p11.15.7 (Pigh) clones described above; a glycine receptor a2 clone (Glar-2; the kind gift of S. Kinsman, University of California at San Francisco, San Francisco, CA); the anonymous DNA clones 45 and 52 for DxPas1 and DxPas2, respectively;31 pMA5-5 (Ang);23 D17Sel4, and Mpr-rs2; and D12Nyu3 and D12Nyu4.23

Gene mapping strategy. To determine the chromosomal location of the genes involved in GPI-anchor biosynthesis, we analyzed a panel of DNA samples from a mouse interspecific backcross that has been characterized for over 700 genetic markers throughout the genome. The genetic markers included in this map span between 50 and 90 centi-Morgans (cM) on each mouse autosome and the X chromosome (for example, see Watson et al). Initially, DNA from the two mouse species was digested with various restriction endonucleases, and hybridized with each cDNA probe to determine RFLVs. Informative RFLVs were identified for each cDNA probe in the SC mice, as compared with the parent CC mice, and these RFLVs were used in gene mapping and linkage analysis (see Fig 1).

After an informative RFLV was identified between the two mouse species for each GPI-anchor gene, it was possible to follow the meiotic segregation of each gene in offspring mice. The parent strains of mice (CC and SS) were bred, and their F1 descendants (designated SC) were then mated with the CC parent strain. The progeny of this backcross mating have random meiotic crossover events in their genome that can be followed by the RFLV. Each backcross mouse was analyzed at the GPI-anchor gene loci for either the homozygous CC pattern or the heterozygous SC pattern. By comparing the haplotype distribution of meiotic crossover events for each gene in a large pedigree of backcross mice, the locus of the gene was mapped relative to other known genes.

Linkage analysis. Gene linkage was determined by segregation analysis.26 Gene order was determined by analyzing all haplotypes and minimizing crossover frequency between all genes that were determined to be within a linkage group. This method resulted in determination of the most likely gene order.

RESULTS

Identification of RFLVs. The parents of the backcross mating (parent strain [CC] and F1 animals [SC]), were analyzed at each GPI-anchor gene locus to identify RFLVs. In each case, one or more hybridization band was present in the SC mice as compared with the CC mice. Southern blots showing the informative RFLVs for clones Piga, Pigf, and Pigh are shown in Fig 1. For Piga, the informative RFLV was 6.0 kb; for Pigf, 5.2 and 19.0 kb; and for Pigh, 4.3 kb.

Analysis of meiotic segregation events. Hybridization of the Piga probe on backcross progeny showed three patterns (data not shown). These three patterns corresponded to both the CC and SS parental patterns and to the F1 (SC) pattern. This result indicated that the Piga gene was located on the
X chromosome, because for autosomal genes, only the CC and SC patterns should be observed. Consistent with this implication, the M. spretus pattern was observed only in male progeny, and the F₁ pattern only in female progeny.

We then used the RFLV between mouse species to follow the meiotic crossover events in the backcross progeny. Comparison of the haplotype distribution, which identifies and quantitates these meiotic crossover events, indicated that in all backcross mice examined, the Piga locus cosegregated with the anonymous DNA locus DxFpasI and with the locus for the glycine receptor α-2 subunit (Glrα-2; Fig 2A). This observation indicated that Piga is very closely linked to these two loci, both of which have been previously mapped to the distal portion of the X chromosome.²⁰,²¹ In contrast, several crossover events were noted between Piga and two other loci, DxFpas2 and Amlg (Fig 2A). For example, the CC haplotype (■) was found in 53 mice, and in 41 mice, the SC pattern (□) was found, indicating no meiotic crossover events. However, in a total of 19 mice (6 + 13), a crossover occurred between the DxFpas2 locus and Piga, whereas 1 mouse (0 + 1) had a crossover between Piga and Amlg. These observations localized the Piga locus between these flanking markers. The best gene order²³ among other distal mouse X chromosome loci ± the standard error²⁴ indicated the following relationships: (centromere)—DxFpas2—16.7 cM ± 3.5 cM—Piga/Glrα-2/DxFpasI—0.9 cM ± 0.9 cM—Amlg. Because there were no crossovers between Piga, Glrα-2, and DxFpasI, these markers are located within an interval of 3.1 cM (upper 95% confidence limit, as determined by binomial distribution).

Hybridization of the Pigm and Pigh probes on appropriately restricted DNA from the backcross progeny showed only two patterns (data not shown). These corresponded to the two parents used in the backcross, CC and SC, consistent with autosomal assignments for these genes (see Table I for informative RFLVs). Comparison of the haplotype distribution of these genes with markers typed throughout the mouse genome mapped the Pigm locus to distal mouse chromosome 17 (Fig 2B). For a total of 107 mice (37 + 70), no meiotic crossover events were noted. However, 6 meiotic crossover events (1 + 5) were noted between D17Sel4 and Pigm, whereas only 1 event occurred between Msrp-rs2 and Pigm. This observation placed the Pigm locus closer to the Msrp-rs2
gene than to D17Sel4. The best gene order ± the standard error was chromosome 17: (centromere)—D17Sel4—5.3 cM ± 2.1 cM—Mrp-rs2—0.9 cM ± 0.9 cM—Pigf.

Similarly, comparison of the haplotype distribution mapped the Pigh locus to the central region of mouse chromosome 12 (Fig 2C). For 96 mice, no meiotic segregation events were identified. For 16 mice, crossovers occurred between D12Nyu3 and Pigh, whereas 2 mice had crossover events between Pigh and D12Nyu4. The best gene order ± the standard error was chromosome 12: (centromere)—D12Nyu3—14.0 cM ± 3.3 cMW—Pigh—1.8 cM ± 1.2 cM—D12Nyu4.

DISCUSSION

In this study, we have used a mouse interspecific backcross mapping technique to assign the chromosomal location of three genes involved in GPI-anchor biosynthesis, namely Piga, Pigf, and Pigh. The mouse Piga locus was mapped to a position on distal mouse X chromosome. Genes that map to the X chromosome in one mammalian species also map to the X chromosome in other mammalian species. In addition, the mapping of Piga was coincident with the position of Glra-2 for which the homologous human locus (GLRX2) has been mapped to the human X chromosome bands p22.1-p21.3.28 Additional genes that flank the Glra-2 locus in the mouse, hypophosphatemia (Hyp), pyruvate dehydrogenase E1a subunit (Pdh1a-1), and amelogenin (Amg) also have human homologs that map to the same region of the human X chromosome: Xp22.2-Xp22.1, Xp22.1, and Xp22.3-Xp22.1, respectively.28,29 Thus, our results strongly suggest that the human Piga gene is located on the short arm of the X chromosome at the Xp22.1 region. These findings are in agreement with the recent report by Takeda et al13 who used fluorescent in situ hybridization to map Piga to human Xp22.1.

In contrast, the Pigh and Pigf genes mapped to regions of mouse chromosomes 12 and 17, respectively. The position of Pigh strongly suggests that the human homolog of this gene will map to the long arm of human chromosome 14 because surrounding genes on mouse chromosome 12 have been mapped to the human chromosome 14 bands q11-24.31 It is less clear where the human homolog of Pigf is likely to be positioned, because conserved linkage relationships for this region of the mouse genome are not well-characterized. The nearest known homologous region would suggest that Pigf will map to the short arm of human chromosome 2. However, it is clear that the human homolog for Pigf will be located on an autosomal chromosome because, as noted above, there are no examples of genes that are autosomes in one mammalian species mapping to the X chromosome in another mammalian species.

These results offer insight into the pathogenesis of PNH. Several lines of evidence, including analysis of glucose-6-phosphate dehydrogenase allozymes25 and a monoclonal pattern of X chromosome inactivation using a hypoxanthine phosphoribosyl transferase probe,33 have previously documented that PNH is a clonal abnormality of bone marrow-derived cells. The acquired enzymatic defect in GPI biosynthesis suggests a dominant mutation within a hematopoietic progenitor cell that affects all of its progeny. Because PNH occurs in both sexes equally, possible explanations would include dominant negative mutations of an autosomal gene, such as that reported for the murine c-kit locus,34 parental imprinting of DNA that results in preferential expression of only one allele,35 or location on the X chromosome with a single allele in males and random inactivation of one allele in females. The assignment of Pigf to the X chromosome clarifies this point and is consistent with the hypothesis that a single gene mutation within Pigf could cause the PNH phenotypic defect. Several recent reports have confirmed that single genetic mutations within the Piga gene can be identified in patients with PNH.16-18

Our findings also help to explain why all patients with PNH tested to date have a class A defect in the GPI-biosynthetic pathway. Although defects in GPI-anchor biosynthesis could result from mutations in other genes along the enzymatic pathway, the location of these other loci on autosomes suggests that both alleles would require mutations to produce clinically overt disease. Our assignment of Pigf and Pigh to autosomes strongly supports this hypothesis; however, there are several additional genes in the GPI-anchor biosynthetic pathway that have not yet been cloned. The chromosomal assignment of these other genes will lead to a better understanding of the pathogenesis of PNH. We predict that all other genes involved in GPI-anchor biosynthesis, like Pigf and Pigh, will map to autosomes.

ACKNOWLEDGMENT

We would like to thank Dr P. Avner for the clones for DxPas1 and DxPas2, Dr M.L. Sneed for the Ang clone, and Dr S. Kinsman for the Gira-2 clone. We gratefully acknowledge J.M. Rochelle for excellent technical assistance, D.C. Bennett and K. Greenwell for secretarial assistance, and Dr Wendell F. Rosse for helpful discussions.

REFERENCES

Chromosomal assignment of genes involved in glycosylphosphatidylinositol anchor biosynthesis: implications for the pathogenesis of paroxysmal nocturnal hemoglobinuria

RE Ware, TA Howard, T Kamitani, HM Change, ET Yeh and MF Seldin