X-Linked Syndrome of Polyendocrinopathy, Immune Dysfunction, and Diarrhea Maps to Xp11.23-Xq13.3

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Summary

We describe genetic analysis of a large pedigree with an X-linked syndrome of polyendocrinopathy, immune dysfunction, and diarrhea (XPID), which frequently results in death during infancy or childhood. Linkage analysis mapped the XPID gene to a 17-cM interval defined by markers DXS8083 and DXS8107 on the X chromosome, at Xp11.23-Xq13.3. The maximum LOD score was 3.99 (recombination fraction0) at DXS1235. Because this interval also harbors the gene for Wiskott-Aldrich syndrome (WAS), we investigated mutations in the *WASP* gene, as the molecular basis of XPID. Northern blot analysis detected the same relative amount and the same-sized *WASP* message in patients with XPID and in a control. Analysis of the *WASP* coding sequence, an alternate promoter, and an untranslated upstream first exon was carried out, and no mutations were found in patients with XPID. A C→T transition within the alternate translation start site cosegregated with the XPID phenotype in this family; however, the same transition site was detected in a normal control male. We conclude that XPID maps to Xp11.23-Xq13.3 and that mutations of *WASP* are not associated with XPID.

Introduction

In 1982, Powell et al. described an X-linked immunodeficiency syndrome associated with autoimmune enteropathy, polyendocrinopathy, atopic dermatitis, and fatal infections. T-cell activation was demonstrated by HLA-DR expression (i.e., circulating lymphocytes and CD8+ T cells infiltrating the gut) without abnormalities of CD25, TCR alpha/beta, or TCR gamma/delta expression (Shigeoka et al. 1993). The clinical characteristics of affected males in this pedigree included eczema, recurrent infections, and autoimmune manifestations. Some of these features are shared with Wiskott-Aldrich syndrome (WAS) (OMIM 301000) (Perry et al. 1980; Sullivan et al. 1994; Ochs 1998), an X-linked disorder with thrombocytopenia, small platelets, eczema, and immunodeficiency. However, this syndrome, which we designate as "X-linked polyendocrinopathy, immune dysfunction, and diarrhea" (XPID), is clinically differentiated from WAS by a lack of platelet defects and may represent a T-cell–activation syndrome. The characteristics of the two disorders are listed in table 1.

Mutations in the WAS gene (*WASP*) are associated with WAS (Derry et al. 1994; summarized by Zhu et al. 1997; reviewed by Nonoyama 1998). Mutations in *WASP* have also been found in patients with X-linked thrombocytopenia (XLT) (MIM 313900), yet these mutations are located at sites different from those described in patients with WAS (Villa et al. 1995; Zhu et al. 1995; De Saint Basile et al. 1996). Therefore, XLT and WAS are allelic disorders resulting from mutations in the same gene, with XLT having a milder phenotype and commonly resulting from *WASP* missense mutations within the first three exons (Zhu et al. 1997). The locus for WAS and XLT maps to Xp11.22-Xp11.23. After initial isolation of *WASP* by Derry et al. (1994), Hagemann and Kwan (1999) characterized its primary promoter and isolated a secondary upstream promoter with an associated untranslated exon. Since some features of WAS and XPID are shared, we hypothesized that XPID might map to Xp11.22-Xp11.23 and might result from mutations in *WASP,* despite the fact that XPID does not share with WAS the common feature of thrombocytopenia or small platelets.

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Table 1

Clinical Features of WAS versus Those of XPID

Clinical Feature	XPID	WAS
Abnormal bleeding	Yes	Yes
Autoimmune manifestations	Yes	Yes
B-cell involvement	No	Yes
Diarrhea	Yes	Yes
Eczema	Yes	Yes
Recurrent infections	Yes	Yes
Small platelets	No	Yes
Thrombocytopenia	No	Yes
T-cell activation	Yes	No

Subjects and Methods

Description of Pedigree

We studied a five-generation Mormon pedigree (K1768; fig. 1) of northern-European ancestry (Powell et al. 1982). No consanguinity was present. The apparent mode of inheritance was X-linked recessive with a history of 20 affected males, 18 of whom had died of severe infections at age ⩽4 years. Of the remaining two patients, one died at age 30 years, the other at age 8 years. Under a protocol of informed consent (University of Utah Medical Center), 15–20 cc of blood was obtained by venipuncture, for isolation of DNA and establishment of permanent cell lines.

Genetic Analysis

Two-point LOD scores were calculated between polymorphic markers and the XPID locus, by means of the computer program LINKAGE (version 5.1) of Lathrop et al. (1985). LOD scores (Z) were calculated under a model of X-linked recessive inheritance. Penetrance was taken as 1.0 for hemizygous males. The frequency of the mutant allele was taken as .0001. For multipoint linkage analysis, the location of the XPID gene was tested against a fixed map of 13 markers (DXS337, DXS8083, DXS426, DXS6941, DXS1126, DXS1039, DXS255, DXS1204, DXS1199, DXS7132, DXS1235, DXS8107, and DXS441), and genetic distances were taken from Généthon.

Reverse Transcription (RT)-PCR Primers for WAS cDNA Synthesis

Several RT-PCR primer pairs were designed for use with *WASP* (Derry et al. 1994). Pairs that gave successful amplification are shown in table 2.

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from Epstein-Barr virus–transformed B lymphocytes derived from two patients (fig. 1) and from an unrelated control, by means of Tri Reagent (Molecular Research Center). Polyadenylated RNA was isolated, by use of a column of oligo (dT) cellulose (Stratagene), from total RNA. Polyadenylated RNA (2 mg) was denatured with glyoxal (12 mM Tris-acetate pH 7.0, 0.3 mM EDTA, 1 M glyoxal) at 50°C for 30 min, size fractionated on a 1% agarose gel, blotted onto a nylon membrane, UV cross-linked, and hybridized at 60°C overnight with ^{32}P-labeled probe. The probe used was a PCR product obtained with primers 5.16 (5′-GGT TTT TTC ATT TCC TGT TC-3′) and 5.17 (5′-CTT GAA GCT ATG GAC ACA TAT G-3′) (Derry et al. 1994), which amplify *WASP* exons 1 and 2 and intron 1 (GenBank accession number AF115549). To control for amounts of RNA loaded, levels of *WASP* message were determined by quantitative methods (PhosphorImager; Molecular Dynamics) and were expressed as a ratio with respect to β-actin–gene RNA level.

DNA Sequencing

Mutation-detection analysis of *WASP* was done by direct sequencing. *WASP* was amplified in two segments. A proximal section was amplified from genomic DNA, and a distal segment was amplified from mRNA. Exons 1 and 2 (including intron 1) were amplified from patient and control genomic DNA, with the primer pair 5.16 and 5.17 mentioned earlier (Derry et al. 1994). For exons 3–12, cDNA was synthesized from total RNA, with viral reverse transcriptase followed by amplification by PCR (RNA PCR kit; PE Biosystems). For 35 cycles of PCR, the cycling parameters were 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Several pairs of RT-PCR primers were designed to amplify the cDNA sequence of WAS (Derry et al. 1994) and are shown in table 2. Amplified products were purified by use of Wizard PCR preps (Promega). DNA sequencing was performed with an ABI373A sequencer by use of the method of dideoxy sequencing by ^{35}S-dATP incorporation. Sequences were analyzed by means of the Sequence Analysis software package (Genetics Computer Group).

Primers WAS-F (5′-ATT GCC AGC TCG TGT GCG GG-3′) and WAS-R (5′-CCT CAG CCA CCA CCA ACT C-3′) were designed to encompass the *WASP* alternate promoter and untranslated exon (Hagemann and Kwan 1999) (GenBank accession number AF115548). Sequencing was performed in both directions, by the method of cycle sequencing by means of [γ-^{33}P]-dATP primer end labeling, according to the manufacturer's specifications (Epicentre Technologies). Thermal cycling was performed on an MJ Researcher PTC-100, with the following conditions: 1 cycle at 95°C for 5 min and 30 cycles at 95°C for 30 s and at 70°C for 1 min. Labeled samples were resolved by electrophoresis through 6%

polyacrylamide gels. Autoradiography was performed for 24–48 h.

RFLP Analysis of the WASP *Alternate Promoter*

A 474-bp amplification fragment containing the *WASP* alternate promoter and untranslated first exon was generated with the primer pair WAS-F and WAS-R. The 25-μl amplification reaction contained ~75 ng genomic DNA, 10 pmol each primer, 1 × Bioline buffer (Bioline), 0.875 U Biolase DNA polymerase (Bioline), 200 μM each dNTP, and 15% glycerol. The thermal profile was 1 cycle at 94°C for 3 min; 31 cycles at 94°C for 30 s, 66°C for 55 s, and 72°C for 1 min; and a final extension cycle at 72°C for 5 min.

PCR gel-extraction purifications were performed with QIAquick gel extraction–purification columns, according to the manufacturer's specifications (Qiagen), and were eluted into 40 μl of 10 mM Tris Cl, pH 8.5. Digestion reactions consisted of ~500 ng of the DNA fragment discussed above, 5 U *Nsp* I (NE Biolabs), 2 μl 10 × digestion buffer, and H_2O double-distilled to 20 μl, incubated for 3 h prior to heat denaturation at 65°C for 20 min. Resulting fragments were resolved on 2% agarose gels and were visualized under UV light after ethidium bromide staining.

Table 2

RT-PCR Primers for *WASP* cDNA Synthesis

Primer[a]	Sequence	Product Size (bp)
273-F	5′-AGA AGT CCT ACT TCA TCC GCC-3′	
846-R	5′-AAC AGA CTC CGC AGA TCT GG-3′	574
653-F	5′-ATC ACG AGT TCA CGA TAC CG-3′	
1469-R	5′-TGC TTC TCT TCT GCA TCA CG-3′	817
1355-F	5′-GGA ATT CAG CTG AAC AAG ACC-3′	
1692-R	5′-GGC AGG GAT AAC AGC ATT-3′	338

[a] Numbers indicate the positions of the first 5′ nucleotides of the primers; "F" and "R" denote forward and reverse orientation, respectively. Every two lines constitute a primer pair.

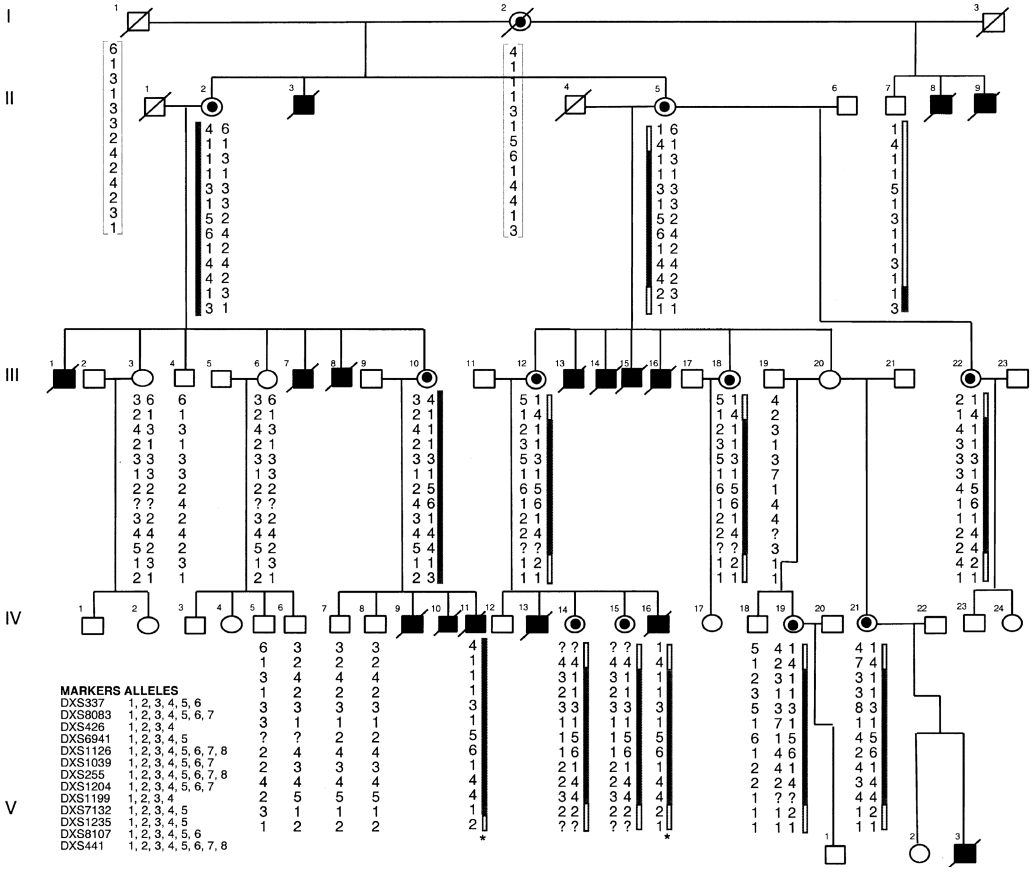

Figure 1 K1768, a five-generation pedigree segregating an X-linked gene for XPID. Two affected males used for northern blot analysis are indicated by asterisks (*). Haplotypes for markers within the X-chromosome bands Xp11.23–Xq13.3 are shown. A crossover distal to marker DXS8083 in individual II-5 defines a proximal flanking marker for XPID. A second recombination seen in the same individual (II-5) and in II-7 occurred distal to DXS1275, defining DXS8107 as the distal flanking marker for the XPID gene region.

Table 3

LOD Scores at Various Recombination Fractions, for Markers on the X Chromosome, in Pedigree K1768

	LOD Score at Recombination Fraction of							Maximum Recombination	Maximum LOD
Marker	.0	.01	.05	.1	.2	.3	.4	Fraction	Score
DXS337	−∞	2.06	2.53	2.54	2.20	1.64	.91	.08	2.56
DXS8083	−∞	1.00	1.53	1.61	1.43	1.05	.54	.11	1.65
DXS426	3.35	3.30	3.11	2.85	2.28	1.62	.86	.00	3.35
DXS6941	1.62	1.60	1.52	1.41	1.15	.84	.46	.00	1.62
DXS1126	1.81	1.78	1.65	1.49	1.13	.75	.37	.00	1.81
DXS1039	1.46	1.44	1.35	1.23	.96	.67	.33	.00	1.46
DXS255	3.81	3.75	3.49	3.15	2.41	1.59	.74	.00	3.81
DXS1204	3.34	3.28	3.05	2.74	2.07	1.34	.64	.00	3.34
DXS1199	2.73	2.70	2.55	2.35	1.86	1.27	.59	.00	2.73
DXS7132	1.81	1.78	1.65	1.49	1.13	.75	.37	.00	1.81
DXS1235	3.99	3.92	3.64	3.28	2.50	1.65	.76	.00	3.99
DXS8107	−∞	.93	1.48	1.58	1.44	1.10	.62	.10	1.58
DXS441	−∞	-1.55	−.26	.19	.47	.46	.29	.24	.49

Results

Linkage Analysis

Two-point disease-to-marker linkage analysis, with markers spanning a 50-cM interval of the X chromosome, detected linkage to Xp11.23-Xq13.3 (fig. 1). A LOD score of 3.35 with no recombination for marker DXS426 established linkage of XPID to this region. Further analysis with 12 additional markers in Xp11.23-Xq13.3 revealed several markers showing significant LOD scores with XPID (table 3). Results for multipoint linkage analysis for all 13 markers analyzed are shown in figure 2.

The estimated genetic distance (17 cM) of the candidate interval of XPID is shown in figure 3. A crossover occurring between markers DXS8083 and DXS426 (individual II-5) defines the proximal boundary of the XPID interval (figs. 1 and 3). A second crossover, detected in the same individual (II-5) and in individual II-7 and located distal to marker DXS1275 and proximal to DXS8107, defines the distal boundary of the XPID region (figs. 1 and 3).

Northern Blot Analysis

A transcript of 2.1 kb was detected in lymphoblastoid cell lines from two patients with XPID and from a control (fig. 4). In addition, when the amount of *WASP* mRNA was expressed as a ratio to β-actin message, a ratio of ~0.1 was obtained for both patients and the control cell line. These observations suggest that, in lymphoblasts, no grossly detectable abnormalities such as deletions or duplications are present in the *WASP* message for XPID patients and that the *WASP* message is present at the same level as that found in a control cell line.

DNA Sequence Analysis

DNA sequence analysis for the *WASP* coding region was performed in two XPID patients and was compared with the normal sequence (Derry et al. 1994). Identical sequences were obtained in the two patients with XPID and in the control.

DNA sequence analysis for the untranslated, alternately spliced first exon, including 22 bp of putative intron 1 and 263 bp of sequence upstream of the putative alternate transcription start site, was performed in two patients with XPID and a control and was compared with that of normal persons (Hagemann and Kwan 1999). Bidirectional sequencing for each of the two samples from affected males detected a C→T transition 7 bp distal to the *WASP* alternate translation start site (*WASP* +7 bp). This transition generates a single recognition site (RCATG/Y) for *Nsp*I.

A 474-bp *WASP* fragment containing the alternate promoter and untranslated first exon was amplified and digested with *Nsp*I. When the *Nsp*I site is present, the restriction digest results in two fragments, of 287 bp and 187 bp (fig. 5). Samples from 24 family members of pedigree K1768 were analyzed for the presence of the *Nsp*I site. The *Nsp*I site showed complete cosegregation with XPID. DNA from 128 unrelated persons was tested for the presence of the *Nsp*I site. The *Nsp*I site was found in 1 of 214 X chromosomes (a normal male), which suggests that it is a rare single-nucleotide polymorphism and not causal for XPID.

Discussion

We mapped the gene for XPID to a 17-cM interval defined by the markers DXS8083 and DXS8107 on the X chromosome (Xp11.23-Xq13.3). The pedigree with

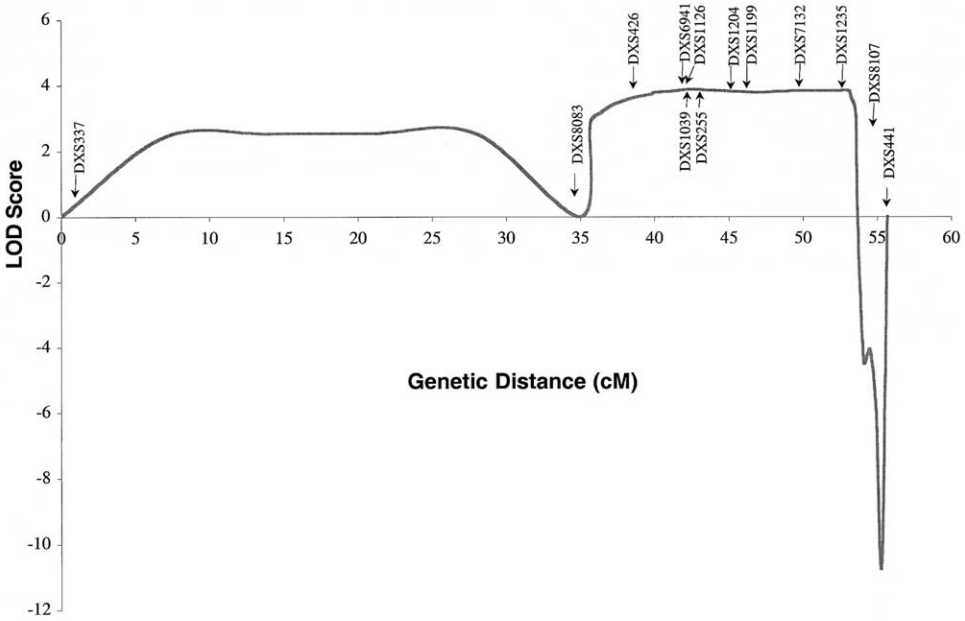

Figure 2 Multilocus analysis, with the XPID gene against fixed genetic map of linked markers (Généthon). The highest probability for location of XPID is in the region DXS426–DXS1235.

XPID described in this report has clinical features that overlap with those of WAS, and the locus for XPID maps to the same region as does the locus for WAS. We thought it essential to examine this gene as a possible candidate for XPID, particularly in light of the recent identification of the alternate transcript for which true temporal and spatial expression is not well understood.

The WASP protein has a complex structure with several distinct functional domains and is putatively involved in the regulation of actin polymerization in hematopoietic cells (Hagemann and Kwan 1999), interacting with several proteins involved in the regulation of signal transduction and cytoskeletal organization (reviewed by Kirchhausen 1998). *WASP* mutations leading to WAS generally lead to a nonfunctional protein and include frameshifts, splice mutations, nonsense mutations, and microdeletions. Conversely, *WASP* mutations leading to XLT, a milder disorder known to be allelic to WAS, are often missense mutations found in exons 1–3 and inhibit less important functions (Zhu et al. 1997). We hypothesized that a particular subset of *WASP* mutations might also lead to XPID. However, in addition to the identification of a correct in-frame coding sequence and correct alternate promoter sequence for *WASP,* we also found the same-sized and relative amounts of *WASP* transcript in patients with XPID and in a normal control. These observations suggest that XPID and WAS are unlikely to be allelic disorders and implicate the existence of another gene, within this region of the X chromosome, related to immune function.

A C→T transition (*WASP* +7 bp) identified in this pedigree with XPID predicts the generation of an ATG start codon leading to a frameshift mutation. We explored the possibility that this C→T transition could result in XPID. However, the same transition was found in a normal control male, which suggests that it is not causal for XPID.

In addition to the lack of platelet abnormalities, XPID is clinically distinct from WAS immunologically in that serum IgM levels are not decreased, isohemagglutinin titers are normal, and there is no elevation of TCR gamma/delta on circulating T cells (Shigeoka et al. 1993). Furthermore, as was not the case for WAS, females heterozygous for XPID show no apparent clonal selection against cells carrying the mutant gene on the active X chromosome (Shigeoka et al. 1993).

Apart from *WASP,* the XPID candidate region (DXS8083–DXS8107) is characterized by several expressed-sequence tags, many of which have no significant homology to transcripts with known functions. An X-linked combined immunodeficiency disorder has been described in which abnormalities are detected in the number and function of T lymphocytes and in the formation of specific IgG antibodies (Brooks et al. 1990), yet this syndrome results from defects of the IL2RG gene (Tassara et al. 1995). The IL2RG gene is excluded as a candidate for XPID, since it maps to a radiation-hybrid bin defined by the markers DXS983–DXS995, distal to the XPID flanking marker DXS8107 (GeneMap'99). Although no other characterized genes in this interval are

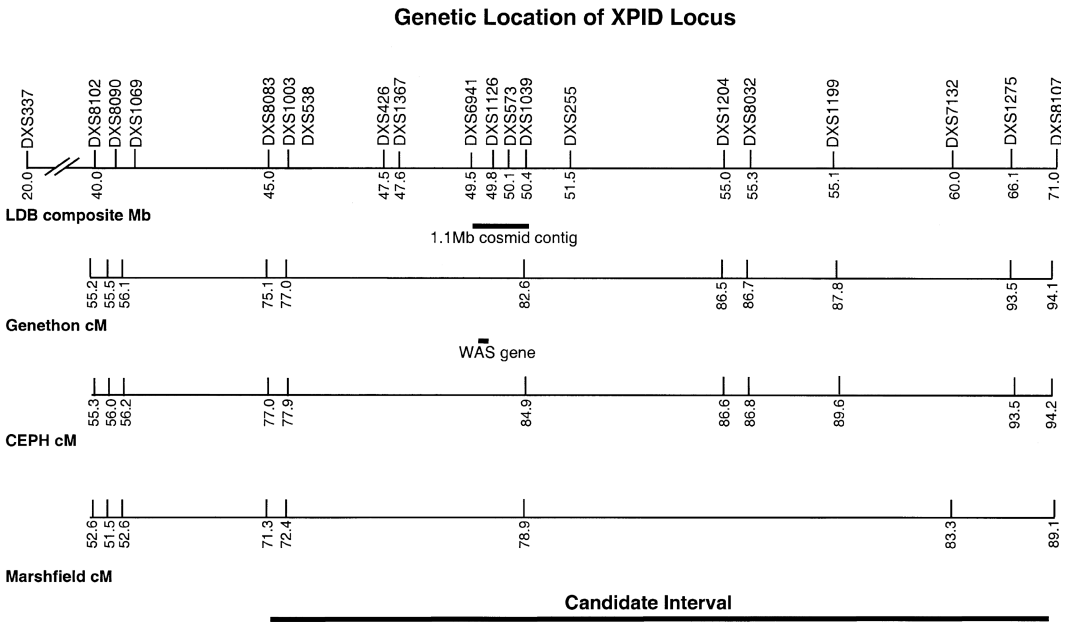

Figure 3 Genetic localization of the XPID locus to Xp11.23–Xq13.3. The horizontal bar indicates the XPID candidate interval at ~17 cM averaged over the three genetic maps of CEPH (Fondation Jean Dausset/CEPH), Généthon, and the Center for Medical Genetics, Marshfield Medical Research Foundation. The relative location of a 1.1-Mb sequence-ready cosmid contig (Schindelhauer et al. 1996) is indicated at markers DXS6941–DXS1039, a region also harboring *WASP*.

obvious functional candidates for XPID, this interval may harbor the human homologue of the mouse scurfy gene. In 1990, it was suggested that a mouse mutant known as "scurfy" may be a model of human WAS (Lyon et al. 1990). Interestingly, the scurfy mutation (sf) maps near the mouse homologue of *WASP* (*Wasp*) on the X chromosome (Derry et al. 1995). Thrombocytopenia, eczema, diarrhea, and immune dysfunction are seen in sf hemizygotes (Lyon et al. 1990), raising the possibility that the human homologue for sf is an attractive positional and functional candidate gene for XPID.

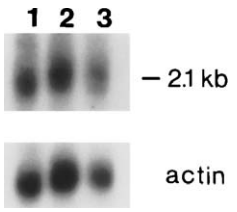

Figure 4 Northern blot analysis of *WASP* in XPID and control RNA. Lanes 1 and 2 contain RNA from B-lymphoblastoid cell lines derived from XPID patients; lane 3 contains RNA control. The probe used was a PCR product generated by amplification of normal genomic DNA with primers 5.16 and 5.17 spanning exons 1 and 2 of *WASP*. A control hybridization with β-actin cDNA is also shown (*bottom*). For autoradiograms, the exposure time for the *WASP* message was 7 d; for the β-actin control, it was overnight. The ratio of hybridization signals for the *WASP* message (2.1 kb) versus the β-actin message is ~0.1, for both patients with XPID and for a control cell line.

Further refinement of the XPID candidate region may be required before a positional-cloning approach to gene isolation is feasible. This will involve the identification of additional families with XPID. Levy-Lahad et al. (1993) documented a family in which two maternal half-brothers had polyendocrinopathy, fatal infection, enteropathy, and thrombocytopenia. Polyendocrinopathy, including diabetes mellitus, enteropathy, and fatal infection, are shared features in both of Levy-Lahad et al.'s patients and in XPID, although the presence of thrombocytopenia suggests that the former syndrome may actually be a variant of classical WAS. Moreover, there are several reports in which infant males had intractable diarrhea with or without diabetes mellitus and renal disease (Ellis et al. 1982; Mitton et al. 1989; Seidman et al. 1990; Colletti et al. 1991). Congenital hypothyroidism and diabetes mellitus were present in one of these infants (Ellis et al. 1982), and an autoimmune mechanism was suggested in the pathogenesis (Ellis et al. 1982; Mitton et al. 1989; Seidman et al. 1990; Colletti et al. 1991).

In conclusion, XPID represents a distinct clinical disorder among the forms of X-linked immunodeficiency syndromes. We have mapped the gene for XPID to a 17-cM interval defined by the markers DXS8083 and DXS441 on chromosome Xp11.23-Xq13.3 and have ex-

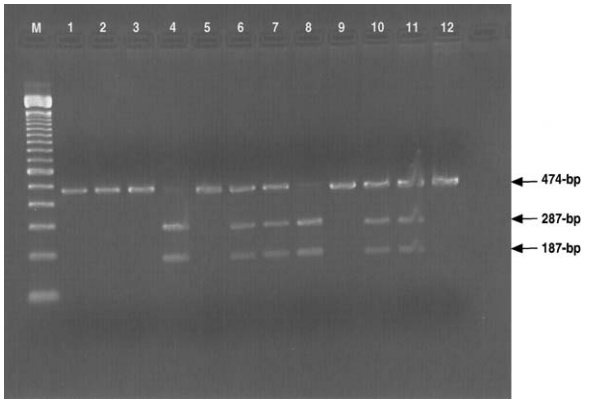

Figure 5 *Nsp*I site analysis of the *WASP* alternate promoter fragment. Lane 1; 700 ng 100 bp marker (Gibco BRL); lanes 1–3, 5, 9, and 12 homozygous *Nsp* I−; lanes 4 and 8 hemizygous *Nsp* I+; and lanes 6, 7, 10, and 11 heterozygous *Nsp* I+/*Nsp* I− carrier females within this XPID pedigree.

cluded mutations in the promoter and coding regions of *WASP* as the molecular basis of this disorder. These findings suggest the existence, within this region of the X chromosome, of another gene influencing immune function.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Fondation Jean Dausset/CEPH, http://www.cephb.fr/cephdb

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html(for *WASP* alternate promoter and untranslated exon [accession number AF115548] and *WASP* exons 1 and 2 and intron 1 [accession number AF115549])

GeneMap'99, http://www.ncbi.nlm.nih.gov/genemap/

Généthon, http://www.genethon.fr

Center for Medical Genetics, Marshfield Medical Research Foundation, http://www.marshmed.org/genetics

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for WAS [MIM 301000] and XLT [MIM 313900])

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