JM2, encoding a fork head–related protein, is mutated in X-linked autoimmunity–allergic disregulation syndrome

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X-linked autoimmunity–allergic disregulation syndrome (XLAAD) is an X-linked recessive immunological disorder characterized by multisystem autoimmunity, particularly early-onset type 1 diabetes mellitus, associated with manifestations of severe atopy including eczema, food allergy, and eosinophilic inflammation. Consistent with the allergic phenotype, analysis of two kindreds with XLAAD revealed marked skewing of patient T lymphocytes toward the Th2 phenotype. Using a positional-candidate approach, we have identified in both kindreds mutations in JM2, a gene on Xp11.23 that encodes a fork head domain–containing protein. One point mutation at a splice junction site results in transcripts that encode a truncated protein lacking the fork head homology domain. The other mutation involves an in-frame, 3-bp deletion that is predicted to impair the function of a leucine zipper dimerization domain. Our results point to a critical role for JM2 in self tolerance and Th cell differentiation.

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Introduction

An X-linked recessive syndrome of autoimmunity, allergic disregulation, and diarrhea has been recognized in several families (1–6) (Mendelian Inheritance in Man entries 304930, 304790). This syndrome, referred to herein as XLAAD (for X-linked autoimmunity–allergic disregulation syndrome), presents early in life with autoimmunity, severe allergic inflammation, secretory diarrhea, and failure to thrive (1–6). Many affected males suffer from classical type 1 diabetes mellitus that frequently presents in the immediate postnatal period or early infancy. Type 1 diabetes mellitus in XLAAD is characterized by islet cell destruction by infiltrating T cells and, in some cases, by autoantibody formation (7). Patients with XLAAD also manifest a more general predilection to autoimmune diseases including autoimmune polyendocrinopathies (especially thyroiditis), hemolytic anemia, thrombocytopenia, and enteropathy. Severe allergic inflammation in XLAAD patients is reflected by the occurrence of eczema, food allergy, elevated IgE levels, and peripheral eosinophilia. Many patients suffer from persistent secretory diarrhea, which may be caused by both food allergy–associated eosinophilic gastroenteropathy and autoimmune enteropathy. Susceptibility to recurrent staphylococcal infections has been variably described in some XLAAD patients and may reflect the well-known association of staphylococcal infections with severe eczema. XLAAD is frequently fatal, due to unremitting diarrhea and wasting, difficult-to-treat diabetes, and/or superimposed infections.

XLAAD has been previously mapped in two unrelated kindreds to Xp11.23–Xq13.3 (6, 8). We have studied two additional kindreds with XLAAD and have mapped the XLAAD locus to an overlapping region on the X chromosome. We report the identification by a positional-candidate approach of a gene encoding a fork head homology domain–related protein that is targeted by mutations in XLAAD patients.

Methods

Clinical material. Peripheral blood samples were obtained for study. Informed consent was obtained from all study participants or their legal guardians.

DNA and RNA isolation. Lymphocytes isolated from whole blood were used to generate phytohemagglutinin-driven (PHA-driven) T-cell lines and Epstein-Barr virus–transformed B-cell lines. DNA was extracted with
Puregene kit (Gentra Systems Inc., Minneapolis, Minnesota, USA), and total RNA was obtained using the Trizol Reagent (Life Technologies Inc., Rockville, Maryland, USA). cDNA was made using AMV–reverse transcriptase (Promega Corp., Madison, Wisconsin, USA).

**Mapping of XLAAD candidate interval.** Genotyping of microsatellite markers in patients and their family members was accomplished using standard PCR primers and methods for the ABI 377 DNA sequencer (Applied Biosystems, Foster City, California, USA). Resulting data were processed through the ABI program Genotyper, to determine marker alleles for each family member. LOD-Score (option of LINKAGE program) was used to determine possible linkage to the X chromosome markers: DXS1223, DXS1068, DXS6810, DXS7132, DXS6800, DXS6789, DXS6797, DXS1047, and DXS7127, which cover an estimated 76 cM interval on the X chromosome on the sex-averaged Marshfield map.

**Candidate gene identification.** Genes in the syntenic human region of the Scurfy critical interval were identified by searching public domain data bases. Homology searches for candidate transcriptional regulators were carried out by BLAST search (www.ncbi.nlm.nih.gov). Two leading candidate transcriptional regulators were identified within the syntenic human region of the Scurfy mouse: Tcfe3, whose deficiency leads to a phenotype distinct from Scurfy (9), and JM2. The gene sequence of JM2 has been previously established as part of the human genome sequencing project (GenBank accession no. AF235097; 997–1176 of NM_014009). The corrected open reading frame sequence is 1146 bp long encoding a 381–amino acid protein.

**Mutation detection.** PCR primers used to amplify individual JM2 exons from genomic DNA and JM2 transcripts from T lymphoblast–derived cDNA are listed in Table 1. PCR amplification of genomic sequences was carried out by heating the reaction mix at 95°C for 1 minute followed by 35 cycles with the following settings: 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Amplified products were sequenced with an ABI 377 sequencer with labeled di-deoxy terminators. Nested PCR amplification of cDNA sequences was carried out using cDNA derived from total RNA of T lymphoblasts by reverse transcription. A first-step PCR reaction was carried out using outer primers (bp 41–59; 1140–1121). PCR conditions were 95°C for 1 minute followed by 35 cycles with the following settings: 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Amplified products were sequenced on an ABI 377 sequencer with labeled di-deoxy terminators.

**Table 1**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>TGATCACACTCTGAGCTCG</td>
<td>GAAGGCAGAGATGCTCACGA</td>
</tr>
<tr>
<td>2</td>
<td>CGTGTGACTCTTTCCCCCTA</td>
<td>ACAGTTAAGGGCTGCACTTGA</td>
</tr>
<tr>
<td>3</td>
<td>CCCACTTACAGGACACTCTC</td>
<td>CGTCTCAGACCCAAGGACCTC</td>
</tr>
<tr>
<td>4</td>
<td>GCCTAAAAGAGACGGCGTTG</td>
<td>CCACATGGACAGACATG</td>
</tr>
<tr>
<td>5</td>
<td>CACCTTCCAAATTCGAGG</td>
<td>TATGTTGAGCTAGGCTGG</td>
</tr>
<tr>
<td>6</td>
<td>GCTTACTCCAGTGTGAGGGTTG</td>
<td>GTTCTGAGAGCTGAGGATTA</td>
</tr>
<tr>
<td>7</td>
<td>GAGTAGGGAGATGCCAAA</td>
<td>CAGTCTGAGTGCGACACAC</td>
</tr>
<tr>
<td>8</td>
<td>GGCCACAAGAGCAGACATC</td>
<td>CCCAGACCTGTGGCAGATA</td>
</tr>
<tr>
<td>9</td>
<td>GTGGACAGGGGTGTTGACGG</td>
<td>GGCACATAGGGAGAGAGG</td>
</tr>
<tr>
<td>Exons 10 and 11</td>
<td>CCCCATCTATGTCCTCTCC</td>
<td>TGGTCGTACATCTCCTTC</td>
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</tbody>
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**Table 2**

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Patients</th>
</tr>
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<tbody>
<tr>
<td>Failure to thrive</td>
<td>5/5</td>
</tr>
<tr>
<td>Eczema</td>
<td>4/5</td>
</tr>
<tr>
<td>Food allergy</td>
<td>5/5</td>
</tr>
<tr>
<td>High IgE levels</td>
<td>4/5</td>
</tr>
<tr>
<td>Type 1 diabetes mellitus</td>
<td>5/5</td>
</tr>
<tr>
<td>Chronic diarrhea</td>
<td>5/5</td>
</tr>
<tr>
<td>Autoimmune cytopenia</td>
<td>3/5</td>
</tr>
</tbody>
</table>

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minute. An aliquot of the first PCR reaction mix was then used as template for the second PCR reaction, which was run under the same conditions except for annealing temperatures of 60°C for primers bp 353–372; 1102–1083 (for detection of exon 7 mutation) and 65°C for primers bp 639–659; 1036–1014 (for detection of exon 9 skipping).

**RNase protection assay.** Peripheral blood mononuclear cells of patients and male controls were stimulated with PHA for 5 days. The lymphoblasts were either left untreated or activated with phorbol ester and calcium ionophore for 1 hour, as indicated. Total RNA was isolated and examined for cytokine expression by RNase protection assay using the Multi-Probe Hck-1 Kit (Pharmingen, San Diego, California, USA).

**Results and Discussion**

We studied two families (referred to as XLAAD-100 and XLAAD-200) with a total of five affected males, one in XLAAD-100 and four in XLAAD-200 (Figure 1). All affected children suffered from type 1 diabetes mellitus with onset in infancy (range 3 weeks to 11 months), chronic diarrhea, and allergic reactions especially to foods (Table 2). Other family members, including obligate heterozygote female carriers of the XLAAD-200 kindred, were clinically unaffected. Laboratory studies revealed evidence of heightened allergic reactivity including eosinophilia and elevated IgE levels, as well as positive radioallergosorbent tests and positive immediate hypersensitivity responses by skin prick tests to food and other allergens. After in vitro stimulation of peripheral blood lymphocytes with mitogens, there was exaggerated expression of the Th2 cytokines IL-4, IL-5, IL-10, and IL-13 and decreased expression of the Th1 cytokine IFN-γ (Figure 2). Both the clinical phenotype and the laboratory studies were consistent with dysregulated Th cell type 2 responses in XLAAD.

The XLAAD susceptibility locus was mapped by...
screening the probands and their relatives with polymorphic markers from the X chromosomes. Two-point linkage analysis mapped the XLAD locus to a 46 cM interval that is flanked by the polymorphic markers DXS1223 proximally and DXS6789 distally, with a lod score of 1.81 at DXS6810 (Figure 1). This interval overlaps those previously defined in other XLAD families. Importantly, the XLAD interval overlaps with the critical interval of the Scurfy mouse gene, a murine model of dysregulated lymphocyte activation (10). Scurfy exhibits several XLAD-related features, including T-cell hyperactivation and enhanced Th2 cytokine production, cytopenia, eczema, and diarrhea (11, 12). This suggested that alterations in the human homologue of the mouse gene responsible for the Scurfy mutation may also be responsible for XLAD.

The critical region of Scurfy has been localized to a ~300 kb segment on chromosome Xp11.23–p11.22 (13, 14). Accordingly, coding sequences in the public domain that map to the syntenic human region were systematically screened for candidate XLAD genes. Given evidence of enhanced Th2 lymphokine gene transcription, particular emphasis was placed on screening either established or candidate transcriptional regulators, the latter identified by BLAST search. JM2, encoding a candidate transcription factor of previously unknown function, was identified in the syntenic human region of the Scurfy critical interval. The JM2 open reading frame is long and is predicted to encode a 381-amino-acid-long protein that contains a fork head homology domain. This is a highly conserved DNA-binding domain that defines the HNF-3/fork...
head family of transcription factors and is characterized by distinct winged helix structure (15, 16) (Figure 3). JM2 is distinguished from other fork head homology proteins by the location of the fork head homology domain at the carboxyl-terminus and by the additional presence of a leucine zipper (Zip) dimerization domain midway through the protein (Figure 3). A putative nuclear localization signal is found at the carboxyl-terminus.

The critical role played by fork head homology and Zip domains in JM2 function was highlighted by the mutations found in the two XLAAD kindreds. Genomic DNA sequencing of the XLAAD-100 proband revealed the presence of an A→G substitution at position +4 of the 5′ donor splice junction of IVS9 (Figure 4a). This substitution was not present in the child’s two other siblings or his parents, including his mother, indicating that it arose de novo. It was also lacking in 100 X chromosomes screened for this mutation. Sequence analysis of RT-PCR-amplified JM2 mRNA transcripts revealed skipping of JM2 exon 9 in transcripts of the index case of family XLAAD-100 but not in unaffected family members or in unrelated controls (Figure 4, b and c). Exon 9 skipping results in a frame shift at codon 273 that gives rise to a premature stop signal at codon 286. This leads to the generation of a truncated JM2 protein that lacks the fork head homology domain (Figure 4d). When the RT-PCR was run at a less stringent annealing temperature (60°C instead of 65°C), a second minor RT-PCR product appeared. This product, which migrated slower than its wild-type counterpart, resulted from aberrant splicing at IVS9 5′ donor splice junction (data not shown). These results confirmed the pathogenicity of IVS9 +4 A→G mutation due to its disruption of IVS9 5′ donor splice junction.

Analysis of the XLAAD-200 kindred revealed affected males to suffer from a 3-bp deletion in JM2 exon 7, resulting in an in-frame deletion of bp 600–602 of JM2 cDNA (Figure 5a). Mothers of affected males, as well as the grandmother, were heterozygous for the mutant allele, while unaffected family members and control subjects lacked this mutation. There was in XLAAD-200 an unexpectedly high incidence of fatal hydrops fetalis. In one tested baby (XLAAD-200-29), the JM2 gene was normal; the others (XLAAD-200-16, -17, -18) were not available for testing.

The mutant transcripts are predicted to encode a JM2 protein lacking glutamic acid 201 residue (ΔE201). This residue lies in the second of the three heptad repeats that constitute the JM2 Zip motif. The JM2 Zip motif is most closely related to the three-heptad Zip motif of N-myc, with virtual identity at the second heptad (17) (Figure 5). Previous studies have revealed an essential role for the N-myc Zip motif and its individual heptad repeats including the second heptad in N-myc homodimerization and in heterodimerization of N-myc with its partner protein Max (18, 19). Specifically, alteration of the N-myc second heptad by mutagenesis of the distal leucine residue to proline or by mutagenesis of the glutamic-lysine-glutamic (EKE) motif to alanine residues impairs homo/heterodimerization and homodimerization, respectively (19). By analogy, it is speculated that the ΔE201 deletion may interfere with heterodimerization of JM2 with partner proteins and/or its homodimerization, resulting in failure of effector function.

Studies on the Scurfy mouse have confirmed that disease pathogenesis is mediated by CD4+ T helper cells (20–22). The disease can be induced by transfer of Scurfy CD4+ T cells to normal hosts, and it is cured upon breeding of Scurfy mice into T cell-deficient
polymorphisms in JM2 may more broadly contribute to the pathogenesis of sporadic type 1 diabetes mellitus. JM2 may also interact with other previously established susceptibility genes for type 1 diabetes mellitus such as HLA-DR genes, a premise suggested by the observation that male bias and linkage to Xp11 in sporadic type 1 diabetes mellitus is most prominent in HLA-DR3+ individuals (28). These issues will need to be addressed in broad-based population studies on sporadic type 1 diabetes mellitus.

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