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Two Mutational Hotspots in the Interleukin-2 Receptor γ Chain Gene Causing Human X-Linked Severe Combined Immunodeficiency

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Summary

Human severe combined immunodeficiency (SCID), a syndrome of profoundly impaired cellular and humoral immunity, is most commonly caused by mutations in the X-linked gene for interleukin-2 (IL-2) receptor γ chain (IL2RG). For mutational analysis of IL2RG in males with SCID, SSCP screening was followed by DNA sequencing. Of 40 IL2RG mutations found in unrelated SCID patients, 6 were point mutations at the CpG dinucleotide at cDNA 690–691, encoding amino acid R226. This residue lies in the extracellular domain of the protein in a region not previously recognized to be significantly conserved in the cytokine receptor gene family, 11 amino acids upstream from the highly conserved WSXWS motif. Three additional instances of mutation at another CpG dinucleotide at cDNA 879 produced a premature termination signal in the intracellular domain of IL2RG, resulting in loss of the SH2-homologous intracellular domain known to be essential for signaling from the IL-2 receptor complex. Mutations at these two hotspots constitute >20% of the X-linked SCID mutations found by our group and a similar proportion of all reported IL2RG mutations.

Introduction

Severe combined immunodeficiency (SCID) is a rare syndrome characterized by profoundly impaired cellular and humoral immune function (Bortin and Rimm 1977; Fischer 1992; WHO Scientific Group on Immunodeficiency 1992; Puck 1993). Without bone marrow transplantation, affected patients suffer severe and persistent infections, usually leading to death in infancy. Both X-linked and autosomally inherited forms of SCID are recognized. The X-linked form, which is the most frequent, is caused by mutations of the gene for the γ chain of the interleukin-2 (IL-2) receptor, IL2RG (Noguchi et al. 1993*a*; Puck et al. 1993*b*). The next most common and longest studied autosomal form is caused by deficiency of adenosine deaminase (ADA) encoded on chromosome 20q12-q13.11 (Hirschhorn 1993; Buckley et al. 1995), but several other known and unknown autosomal gene defects can cause SCID (WHO Scientific Group on Immunodeficiency 1992; Puck 1993).

Identification of IL2RG as the X-linked SCID disease gene has made possible mutation detection in individual patients. More than 20 unique mutations have been reported in the literature by our group (Puck et al. 1993*b*) and by others (Noguchi et al. 1993*a*; DiSanto et al. 1994*a*, 1994*b*; Ishii et al. 1994; Markiewitz et al. 1994; Russell et al. 1994), and further mutational analysis by our group has to date characterized the IL2RG mutations in a total of 40 unrelated males with SCID (Puck 1994*a*, 1994*b*), all of which have consisted of minor DNA sequence alterations. We have found that two mutation sites have repeatedly caused SCID in unrelated individuals. Because IL2RG-associated X-linked SCID is more common than autosomal SCID, these mutational hotspots account for a substantial proportion of all SCID.

Subjects and Methods

Subjects

Males with SCID, both sporadic and in pedigrees showing X-linked inheritance, were ascertained through immunologists and geneticists. Those with ADA deficiency, other known forms of autosomal SCID, or mild phenotypes were excluded. DNA from the unrelated SCID-affected males and their relatives was prepared from samples obtained with informed consent, including blood, cell lines, and tissue from biopsy or autopsy. Forty-four of these families were found to have X-linked SCID, either by maternal carrier status—manifested by skewed T-cell X chromosome inactivation (Puck et al. 1992)—or by finding an IL2RG abnormality by SSCP, altered mRNA expression by northern analysis, or se-

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quence (Puck 1994*a*, 1994*b*). Clinical data and pedigrees were reviewed; relatedness among our families or between patients reported here and those studied by other investigators was not found; patients 3, 10, 14, and 28 were included in previous clinical publications (Barrett et al. 1988; Conley et al. 1990). Specific mutations have been defined in 40 patients (Puck et al. 1993*b*; Puck 1994*b*; and unpublished data).

PCR Method

The eight exons of IL2RG and surrounding genomic sequences were preamplified from genomic DNA by using published primer sequences (Puck et al. 1995) in segments consisting of exons 1–3, 4–6, and 6–8. Reactions contained 10–100 ng of genomic DNA in 10–50 μl with 0.5 μM of each primer, 1.25 U of AmpliTaq polymerase (Perkin Elmer), 200 μmol of each dNTP, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.001% gelatin. A 94°C soak for 30 min was followed by 35 cycles of 94°C for 45 s, an annealing step for 60 s, and 72°C for 120 s (GeneAmp PCR System 9600; Perkin Elmer). The annealing temperature was progressively decreased from 72°C to 60°C over the first 12 cycles, remaining at 60°C thereafter.

SSCP Method

Single exons were amplified with nested primers from the preamplified segments using the PCR conditions above, with one primer end-labeled with γ-^{32}P-ATP (Puck 1994*a*). Primers for exon 5 were 5′-AGTAGCACAGATGACACTGGTGG (coding) and 5′-TAGAAAGGCTGGGGTGTTGG (antisense), amplifying a 312-bp genomic fragment including the 163-bp exon. Primers for exon 7 were 5′-TTTGGTGATGGAAGGAAGCC (coding) and 5′-ACACATCTGTCTGTCTTGCTGGC (antisense), amplifying a 271-bp fragment including the 70-bp exon. PCR products were denatured by boiling, then immediately cooled on ice, and separated on nondenaturing Hydrolink MDE gels (AT Biochem) overnight at 7 W. The gels were dried and autoradiographed. Under these SSCP conditions mutations produced single or multiple bands different from, or in addition to, control bands, each band representing a different, stable single-stranded conformation (reviewed in Hayashi and Yandell [1993]). In cases where DNA strands from a single X chromosome (such as a SCID male) showed multiple migration conformers, their identity was confirmed by direct sequence determination.

Sequencing

Exon 5 and 7 were sequenced using as templates preamplified PCR products, electrophoresed through low-melting temperature agarose and purified with a Qiaex kit (Qiagen) or directly isolated DNA from SSCP gels. A Cyclist DNA sequencing kit (Stratagene) was used with one primer end-labeled with γ-^{32}P-ATP.

Restriction and Genotyping

For restriction analysis exons 4–6 were amplified with the following primers: 5′-GGTATTAGGGGCACTACCTTCAGG (coding) and 5′-ACCCTCCTCTGCTATTGTCAGC (antisense). Restriction digestion with *Aci*I (New England Biolabs) was carried out according to the manufacturer. PCR genotyping at DXS453 and DXS441 was as described (Ram et al. 1992; Puck et al. 1993*a*).

IL-2 Binding

Biotin conjugated human IL-2 and avidin-fluorescein were sequentially bound to patient and control B-cell lines transformed with Epstein-Barr virus, using a Flourokine kit (R and D Systems). Cells were analyzed using a FACSCAN flow cytometer (Becton Dickinson).

Results

Mutation Screening

SSCP performed with primer pairs surrounding each of the eight IL2RG exons and splice sequences (Puck et al. 1995) was used to localize SCID-associated mutations; no more than one abnormally migrating exon was observed in any patient. Specific mutations were then identified by direct sequence of the PCR products of abnormal exons. Although neither magnitude of migration shifts nor number of SSCP bands were predictive of the nature of the mutation, the same altered SSCP patterns were noted to occur in multiple patients.

Mutation at cDNA 690–691

SCID boys 14, 25, and 28 (fig. 1) had identical and distinctive SSCP patterns in IL2RG exon 5, consisting of a slowly migrating band, due to an altered conformation, in addition to a band with the same migration rate as control exon 5 DNA, shown at the right of figure 1. The same abnormal conformational pattern was detected in DNA from boys 32 and 55 (not shown). Although the older brother of boy 32 had died of SCID, the remaining four patients had no family history of the disease (table 1). The mothers of boys 14, 25, and 32 had previously been diagnosed as X-linked SCID carriers on the basis of skewed T-cell X chromosome inactivation ("S," fig. 1) (Puck et al. 1992). SSCP patterns from these women were consistent with heterozygosity for the mutant allele, showing the abnormal conformation at reduced intensity as compared with the normal, as seen in figure 1. Also shown in figure 1 is a somatic cell hybrid segregating the nonmutant, active X chromosome from the mother of boy 14; the hybrid had only the normally migrating band, indicating that the retarded conformational pattern was specific to the mutant IL2RG allele.

A newly arising SCID mutation had been inferred in a prior study for boy 28, because his mother's T-cell X chromosome inactivation had a normal, random pattern

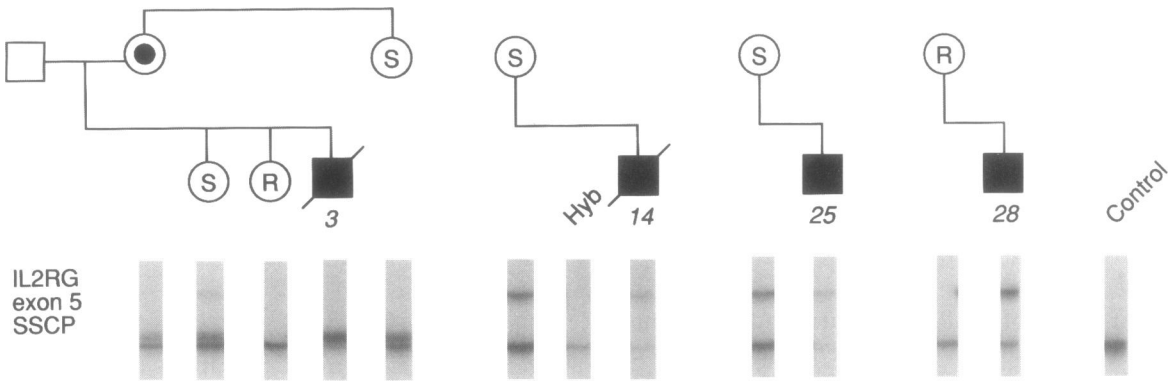

Figure 1 Abnormal SSCP patterns in IL2RG exon 5 in SCID patients 3, 14, 25, and 28, and selected relatives. S = skewed T-cell X inactivation; R = random X inactivation; Hyb = hybrid cell line segregating the nonmutant, active X chromosome from the mother of boy 14.

("R," fig. 1) (Conley et al. 1990). The absence of a mutation in her bone marrow–derived tissues was confirmed by the SSCP pattern in IL2RG exon 5 genomic DNA from her blood. The mother of boy 55 likewise demonstrated random T-cell X inactivation and no SSCP abnormality (not shown).

A different conformational change, with less extreme gel retardation in IL2RG exon 5, was noted in boy 3 and the members of his large X-linked SCID kindred who were either obligate carriers or females with skewed T-cell X inactivation (fig. 1, *left*).

Sequencing IL2RG exon 5 from the above patients revealed that all had point mutations associated with the same CpG dinucleotide, at cDNA 690–691. The specific mutations and their locations relative to the cDNA domains of IL2RG are shown on the left side of figure 2. Boys 14, 25, 28, 32, and 55 had a C_{690}-to-T transition on the coding strand, resulting in substitution of cysteine for R226 (counting amino acid 1 as the initiating methionine). Boy 3 had a related C_{691}-to-T transition on the noncoding strand, changing G_{691} to A on the coding strand and substituting histidine for R226.

Table 1

Clinical and Laboratory Data for Probands and Affected Siblings

Boy Number	Mutation Site (cDNA)	Ethnic Origin[a]	Age at Diagnosis; Outcome[b]	Immunological Parameters at Diagnosis[c]			
				%T-cells[d]	%B-cells[e]	%NK-cells[f]	%NK targets lysed[g]
3	691	C	4 mo; BMT	14	64	5	6 (50, SD 15)
14	690	AA/H	8 mo; died	0	76	6	19 (50, SD 15)
25	690	C	6 mo; BMT	0	91	6	27 (>20)
28	690	C	3 mo; recurrent varicella, BMT	62[h]	22	1	2 (50, SD 15)
32	690	AA	3 wk; BMT	4	42	6	10 (50, SD 15)
32sib	690	AA	18 mo; died	4	21	6	ND
55	690	C	5 mo; died	0	79	4	ND
10	879	C	4 mo; BMT	45[h]	12	ND	ND
45	879	AA	1 mo; BMT	0	100	0	0 (>20)
45sib	879	AA	1 mo; BMT	0		0	0 (>20)
45sib	879	AA	8 mo; died	ND	ND	ND	ND
58	879	C	6 mo; died	0	38	0	ND

[a] C = Caucasian; AA = Afro-American; and H = Hispanic.
[b] BMT = Bone marrow transplant.
[c] ND = not determined.
[d] Percent of lymphocytes with pan-T-cell markers; normal range 65–75.
[e] Percent of lymphocytes with CD20 or surface Ig; normal range 5–10.
[f] Percent of lymphocytes with NK-cell markers CD16 or CD56; normal range 5–15.
[g] NK-cell lysis of target cells at 100:1 effector:target ratio (normal values for testing center).
[h] T-cells found to be of maternal origin.

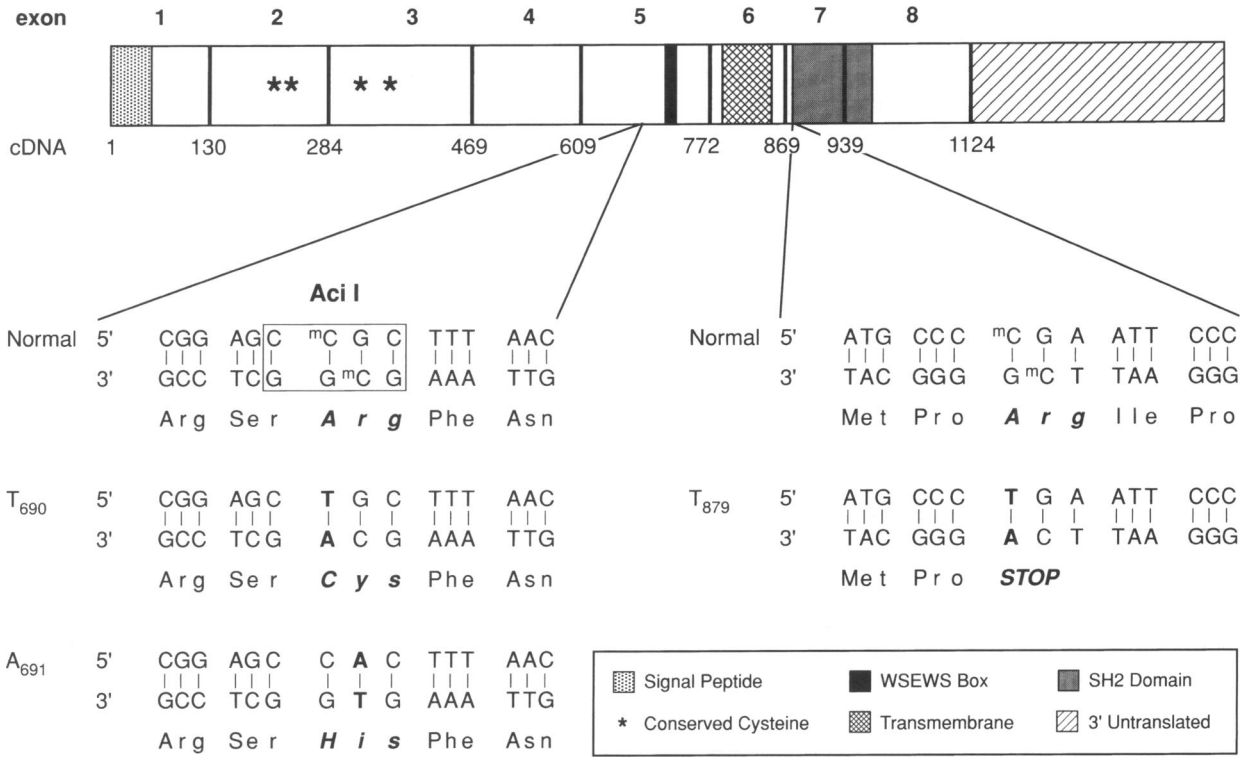

Figure 2 IL2RG cDNA map, showing exon boundaries, protein domains, and two sites of frequent mutation. *Left,* cDNA 690–691 mutations; *right,* cDNA 879 mutation.

The CpG dinucleotide at this location forms the center of an *Aci*I restriction site, CCGC/GGCG (fig. 2, *left*), predicted to be disrupted by mutations at either cDNA 690 or 691. Figure 3 illustrates *Aci*I-digested PCR products from exons 4–6 of IL2RG. Fragments of 1,052 bp and 539 bp were generated with template DNA from a normal individual (fig. 3, *left*). However, DNA from boy 3, with a mutation at cDNA 691, and boys 25, 28 and 32, each with a mutation at cDNA 690, had a fragment of 1,591 bp. The mother of boy 32 (fig. 3, "32M"), a heterozygote for the mutation at cDNA 690, had three bands, representing both digested and undigested fragments by this assay. Similarly, a mixture of independently amplified products from both a normal subject and boy 25, combined before *Aci*I treatment, had both digested and intact fragments (fig. 3, *right*).

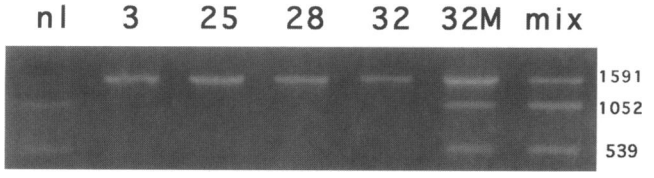

Figure 3 *Aci*I digestion of IL2RG exons 4–6 including cDNA 690–691 for PCR products amplified from normal DNA (*left*); boys 3, 25, 28 and 32; the mother of boy 32; and pooled PCR products from separate amplifications from a normal control and boy 25. Sizes of fragments, in base pairs, are at right.

Mutation at cDNA 879

Multiple unrelated SCID patients were also noted who had the same SSCP migration differences caused by a point mutation at cDNA 879 in exon 7 of IL2RG. Figure 4 illustrates the sequence of exon 7 in boy 10

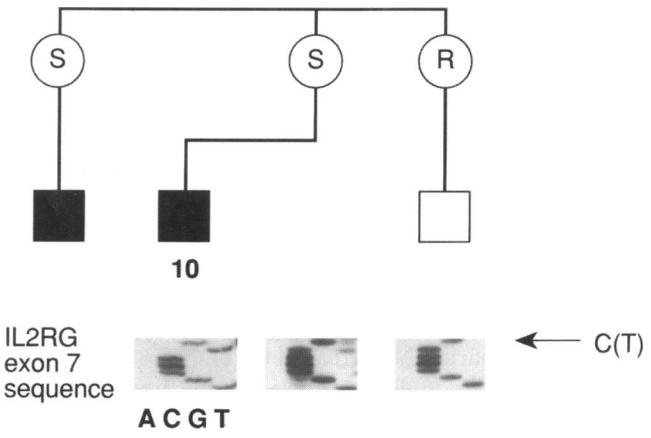

Figure 4 Partial pedigree and sequence analysis of IL2RG exon 7 in boy 10, his obligate carrier mother known to have skewed X inactivation (S), and his unaffected male cousin.

and a portion of his kindred, which included three cases of X-linked SCID in two generations. The wild-type exon 7 sequence from an unaffected male cousin (fig. 4, *right sequence*), had four cytosine residues, the top one forming a CpG dinucleotide. In the sequence of boy 10 (fig. 4, *left*) a C_{879}-to-T transition at this dinucleotide was found. His mother, a SCID carrier both by pedigree analysis and skewed T-cell X chromosome inactivation, had both the normal C and mutant T bands in directly amplified genomic DNA (fig. 4, *center*). Two other males with SCID, boys 45 (one of three affected brothers) and 58, had the identical mutation; their mothers were both shown to be heterozygotes. The position of this mutation on the cDNA map of IL2RG is shown in figure 2 (*right*). The C_{879}-to-T transition produced a TGA termination codon replacing the normal CGA for R289.

Although the cDNA 879 mutation disrupts a restriction site for *Hin*FIII, potentially an aid to rapid diagnosis, this enzyme is not widely available presently. It is noteworthy that boy 58 had died in 1991 of diarrhea and sepsis, prior to bone marrow transplantation, and had not had an autopsy or preservation of tissue for molecular studies. Only a partial paraffin block from a <1 mm^3 intestinal biopsy was available; yet this sample was adequate for PCR-based molecular diagnosis.

Haplotypes of Patients with Hotspot Mutations

Dinucleotide repeat polymorphisms DXS453 and DXS441, known to flank IL2RG closely in proximal Xq (Willard et al. 1994) were used to assess the X chromosomal haplotype of the five patients with cDNA 690 and three patients with cDNA 879 mutations (data not shown). All patients with a given mutation had different DXS453 alleles except two, and one of these, boy 55, was known to represent a new mutation not present in his mother's somatic cells. Similarly, at DXS441, unique alleles were found in all but patients 28 and 55, both of whom had mutations proven genetically to be new.

Genotype-Phenotype Correlations

B-cell derived lines, available for boys 3, 14, 25, and 28 (with mutation at cDNA 690–691) and 10 and 45 (cDNA 879) produced normal amounts of IL2RG mRNA (Puck 1994*b*; and not shown), ruling out unstable message in the pathogenesis of SCID for these mutations. Protein production was evaluated by measuring ability of B-cell lines to bind IL-2 in an immunofluorescence assay (not shown). As might be expected for SCID-causing mutations in the extracellular domain, IL-2 binding was markedly decreased for cDNA 690 and 691 mutant B-cell lines. In contrast, for the lines with the cDNA 879 premature termination in the intracellular signaling domain, IL-2 binding on the cell surface was equal to that of normal control B-cell lines. The entire 4.2-kb IL2RG gene of at least one patient with each mutation was sequenced to rule out other abnormalities, confirming that the CpG point mutations were the cause of SCID.

All patients were ascertained because of severely compromised host defenses, either requiring bone marrow transplantation or resulting in a fatal outcome. Table 1 summarizes for each mutation selected clinical and laboratory data from probands and, for patients 32 and 45, their affected siblings. All presented by 8 mo of age; all had exceedingly low numbers of T-cells, except where transplacental transfer of maternal T-cells had occurred; and all had poor or absent T-cell mitogen responses. Elevated proportions of B-cells, characteristic of X-linked SCID (Schiff and Buckley 1994; Buckley et al. 1995), were seen in all cases, while cells with natural killer (NK) cell–associated markers were often decreased. NK-cell lysis of target cells was poor to absent except in boy 25.

Discussion

The IL2RG mutations at cDNA 690, 691, and 879 are examples of the most common class of mutations in vertebrates, C-to-T transitions within CpG dinucleotides. These single-base substitutions occur at a frequency 42-fold higher than would be predicted from random mutation (Cooper and Krawczak 1993), probably because of methylation of cytosine residues, as depicted in figure 2, followed by deamination, resulting in their being replaced with thymidine.

Evidence that the mutations were newly arising as opposed to the result of a founder effect is manifold: (i) multigeneration X-linked transmission in only two of the nine families; (ii) diverse ethnic and geographic patient origins (table 1); (iii) newly arising mutations proved in two patients with cDNA 690 mutation by showing that maternal somatic cells did not have IL2RG mutations; (iv) haplotype analysis suggesting diverse chromosomal backgrounds; (v) independent reports in the literature of a cDNA 690 (DiSanto et al. 1994*a*) and a cDNA 879 (Noguchi et al. 1993*a*) mutation.

The IL-2 receptor γ chain, also known as the common γ chain, γc, has been shown to participate in multiple cytokine receptor complexes, including the receptors for IL-4 (Kondo et al. 1993; Russell et al. 1993), IL-7 (Noguchi et al. 1993*b*), IL-9 (Russell et al. 1994) and IL-15 (Giri et al. 1994). However, it is not always clear how a particular γc point mutation causes impairment of lymphocyte development and function. Mutations at cDNA 690–691 are single amino acid changes of R226 in an extracellular region of γc not previously associated with a function. However, protein structure algorithms predict this residue to be an antigenic peak on the surface of the protein (Emini et al. 1985; Jameson and Wolf 1988). The introduction of patient mutations R226C

Cytokine Receptor Gene	Amino Acid Sequence Near WSXWS Motif												
IL2RG	QK	RY	T	F	R	VR	S	R	FNPLCGSAQH	WS	E	WS	
			220					226					
EPOR	RT	RY	T	F	A	VR	A	R	MAEPSFGGF	WS	A	WS	
IL12R	VA	Q	E	F	Q	LR	R	R	QLGSQGSS	WS	K	WS	
GMCSFR	EP	R	AKHS			VK	I	R	AADVRILN	WS	S	WS	
IL2RB	DT	QY	E	F	Q	VR	V	K	PLQGEFTT	WS	P	WS	
PRLR	GQ	KY	L	V	Q	VR	C	K	PDHGY	WS	A	WS	

Figure 5 Cytokine receptor superfamily homology in the residues toward the amino terminus of the WSXWS motif. Conserved or closely related residues, shown in single-letter code, are in boldface. The arginine (R) and closely related lysine (K) residues homologous to IL2RG position 226 are underlined.

and R226H in the algorithms changed these predicted properties. R226 is conserved among human, dog, and mouse IL2RG (Henthorn et al. 1994). Moreover, the site is only 11 residues from the absolutely conserved WSXWS motif common to all members of the cytokine receptor family. Inspection of sequences of other cytokine receptors revealed further similarities, as shown in figure 5. The position corresponding to R226, underlined in the figure, is absolutely conserved in the human receptors for erythropoietin (Penny and Forget 1991), IL-12 (IL12R) (Chua et al. 1994), and granulocyte-macrophage colony stimulating factor (Ashworth and Kraft 1990), and relatively conserved with a lysine residue in the IL-2 receptor β chain (IL2RB) (Leonard et al. 1984) and the prolactin receptor (Boutin et al. 1989). As many as six additional highly conserved residues between 218 and 226, shown in boldface in figure 5 suggest that the recurrent point mutations we have observed reflect not only a highly mutable CpG dinucleotide, but also an important domain of γc and other cytokine receptor molecules. Cell surface γc is reduced but is detectable with monoclonal anti-γc antibodies in cell lines with R226 mutations (J. M. Puck, unpublished information). Cell lines from our patients, as well as the R226C mutant patient reported by DiSanto et al. (1994*a*), had decreased IL-2 binding. These findings could be due to decreased amounts of γc protein on the cell surface or alterations affecting the mutant γc IL-2-binding site and antibody recognition epitope.

The mutations at cDNA 879, as first reported by Noguchi et al. (1993*a*), place a termination codon at the position of R289, just six residues to the intracellular side of the transmembrane region of γc and at the beginning of the SH2 domain homologous to src-related kinases. Although surface expression and IL-2 binding are normal, the terminal 81 residues of the protein are predicted to be lost. In vitro mutagenesis and direct analysis of SCID patient cell lines have shown that terminal deletions of this magnitude, as well as smaller ones, ablate downstream signaling by γc (Ishii et al. 1994; Miyazaki et al. 1994; Russell et al. 1994).

Identical mutations in IL2RG do not necessarily produce the same clinical presentation. The X-linked SCID phenotype is generally characterized by high proportions and even high absolute numbers of B lymphocytes, although these lymphocytes fail to produce specific antibodies. As shown in table 1, both T-and B-cell abnormalities of patients with hotspot mutations are typical of X-linked SCID patients. Moreover, our hotspot patients with the lowest, least typical B-cell numbers, boy 10 (156 B-cells/μl) and the deceased brother of boy 32 (74/μl), had different mutations.

Another recognized feature of X-linked SCID is severely depressed NK-cell function (O'Reilly et al. 1994; Schiff and Buckley 1994; Buckley et al., in press). In view of this, we were surprised to learn that boy 25 had the X-linked form of SCID, let alone the same mutation as four other patients, because on presentation he had a normal number of circulating $CD56^+$ NK-cells (180 cells/μl) as well as significant NK function demonstrated by the standard assay of lysis of K562 target cells (table 1). Environmental factors, including age at diagnosis, infections, and the transplacental transfer of maternal lymphocytes into patients with SCID (Conley et al. 1984, 1990; Barrett et al. 1988), may be important contributors to lymphocytic phenotypes in SCID patients. It is possible that the functional NK-cells of boy 25 were transplacentally derived maternal NK cells. Modifying genes, as yet not defined, could also influence the SCID phenotype, including the development of NK cells, in some patients.

While ADA defects account for ~15% of human SCID (Stephan et al. 1993; Buckley et al., in press), a striking male predominance suggests that up to 70% of SCID in the United States may be X-linked because of mutations in IL2RG (Buckley et al., in press). The two mutational hotspots shown here account for 22.5% of the 40 mutations detected by our laboratory and 11 (>20%) of 54 IL2RG mutations in unrelated individuals reported worldwide. Screening for mutations at these two sites through SSCP or digestion with *Aci*I can thus genotypically classify ~15% of all SCID patients, a proportion similar to those with ADA-deficient SCID.

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