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Partial V(D)J Recombination Activity Leads to Omenn Syndrome

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Summary

Genomic rearrangement of the antigen receptor loci is initiated by the two lymphoid-specific proteins Rag-1 and Rag-2. Null mutations in either of the two proteins abrogate initiation of V(D)J recombination and cause severe combined immunodeficiency with complete absence of mature B and T lymphocytes. We report here that patients with Omenn syndrome, a severe immunodeficiency characterized by the presence of activated, anergic, oligoclonal T cells, hypereosinophilia, and high IgE levels, bear missense mutations in either the *Rag-1* or *Rag-2* genes that result in partial activity of the two proteins. Two of the amino acid substitutions map within the Rag-1 homeodomain and decrease DNA binding activity, while three others lower the efficiency of Rag-1/Rag-2 interaction. These findings provide evidence to indicate that the immunodeficiency manifested in patients with Omenn syndrome arises from mutations that decrease the efficiency of V(D)J recombination.

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Introduction

Diversity of the immune repertoire is generated by somatic assembly of the antigen receptor variable gene segments, in a process termed V(D)J site-specific recombination (Tonegawa, 1983; reviewed by Lewis, 1994). This process is dependent on the activation of two key lymphoid-specific proteins, Rag-1 and Rag-2 (Schatz et al., 1989; Oettinger et al., 1990). Recombination is initiated by the specific binding of Rag-1/Rag-2 to the conserved recombination signal sequences (RSSs) that flank each variable coding element (McBlane et al., 1995; van Gent et al., 1995; Difilippantonio et al., 1996; Spanopoulou et al., 1996; Hiom and Gellert, 1997). Each RSS consists of a highly conserved heptamer (CACAGTG) element and a moderately conserved nonamer motif (ACAAAAACC) separated by a spacer of either 12 or 23 nucleotides (Ramsden et al., 1994). Binding to the nonamer element is mediated by a region of Rag-1 that shows distinct homology to the DNA-binding domain of Hin invertases and that of homeodomain (HD) proteins (Difilippantonio et al., 1996; Spanopoulou et al., 1996; Nagawa et al., 1998). Rag-2 alone has no obvious DNA binding activity (Difilippantonio et al., 1996; Spanopoulou et al., 1996; Hiom and Gellert, 1997).

Upon stable binding to the RSS, the Rag-1/Rag-2 complex cleaves the upper strand at the heptamer/coding sequence junction. The cleavage intermediates are a covalently sealed hairpin coding end and a 5′ phosphorylated blunt signal end (McBlane et al., 1995; van Gent et al., 1995). Following the generation of the two double-strand break (DSB) recombination intermediates by Rag-1/Rag-2, several ubiquitous DNA repair activities, including Ku70, Ku80, and DNA-PK are engaged to process the DSB ends (reviewed by Jackson and Jeggo, 1995; Bogue and Roth, 1996; Agrawal and Schatz, 1997). Additional activities such as XR-1 and ligase IV have been suggested to participate at later stages of V(D)J recombination (Grawunder et al., 1997), which finally leads to joining of the coding ends. Although the complete V(D)J reaction has yet to be reproduced with purified components, fully recombined products can be generated in vitro using Rag-1, Rag-2, and total cell extracts (Cortes et al., 1996; Leu et al., 1997; Ramsden et al., 1997; Weis-Garcia et al., 1997).

Rag-1 and Rag-2, as well as the ubiquitous DNA repair activities Ku70, Ku80, and DNA-PK, are all essential for V(D)J recombination. Disruption of Rag-1 or Rag-2 function blocks initiation of V(D)J recombination and leads to complete absence of mature B and T cells (Mombaerts et al., 1992; Shinkai et al., 1992). Ku70-, Ku80-, or DNA-PK-deficient cells fail to resolve the DSB intermediates generated by Rag-1/Rag-2 (Roth et al., 1992; Jackson and Jeggo, 1995; Bogue and Roth, 1996; Nussenzweig et al., 1996; Zhu et al., 1996; Bogue et al., 1997; Gu et al., 1997). Similarly, in humans, mutations that eliminate the recombination activity of Rag-1 or Rag-2 lead to severe combined immunodeficiency (SCID) due to the absence of antigen receptors (Schwarz et al., 1996).

Table 1. Analysis of OS Patients for Mutations in *Rag-1* or *Rag-2*

Patient	Gene	Nucleotide	Amino Acid Change	Domain
OS1	*Rag-2*	C1324G	C41W	HimA homology
		T2055G	M285R	topoII homology
OS2	*Rag-1*	G1794A	R561H	Rag-2 interaction
OS3	*Rag-1*	C1298T	R396C	Homeodomain, DNA binding
		A2847G	Y912C	Active core
OS4	*Rag-1*	G1299A	R396H	Homeodomain, DNA binding
		deletion 1723–1735		Truncated protein
OS5	*Rag-1*	deletion 368–369		No protein
		A1398G	D429G	Homeodomain, Homodimerization
OS6	*Rag-1*	C1298T	R396C	Homeodomain, DNA binding
OS7	*Rag-1*	C1793T	R561C	Rag-2 interaction
		G2322A	R737H	Active core

Omenn syndrome (OS) is a rare autosomal recessive genetic disorder characterized by symptoms of severe combined immunodeficiency associated with erythrodermia, eosinophilia, hepatosplenomegaly, lymphadenopathy, and elevated serum IgE levels (Omenn, 1965; OMIM 267700). Unless treated by bone marrow transplantation, this disease is invariably fatal (Gomez et al., 1995). At variance with other forms of SCID, OS patients have a variable number of circulating T cells that coexpress activation markers and respond poorly to mitogens and antigens. Previous studies have shown restricted heterogeneity of T cell receptor (TCR) rearrangements in patients with OS (de Saint-Basile et al., 1991; Harville et al., 1997). It has been suggested that the clinical and immunological features of OS could be related to an unbalanced expansion of the T helper 2 (Th2) cell subset as substantiated by increased production of interleukins 4 (IL-4) and 5 (IL-5), reduced synthesis of IL-2 and γ-interferon by in vitro activated lymphocytes (Schandené et al., 1993; Chilosi et al., 1996), and increased numbers of CD30-expressing cells, a surface marker preferentially expressed on Th2 cells (Chilosi et al., 1996). The underlying molecular defect remained unknown although similarities with the murine *scid* model have been described (Cavazzana-Calvo et al., 1993).

We report here the analysis of seven OS patients, all of whom were found to bear recessive mutations in either the *Rag-1* or *Rag-2* genes. All OS mutations lead to reduced V(D)J recombination activity and account for the immunodeficiency observed in OS patients. These findings establish V(D)J recombination deficiencies as the molecular basis of OS.

Results

Detection of Rag Mutations in OS Patients

We studied seven patients fulfilling the criteria for Omenn syndrome, i.e., early-onset generalized erythrodermia, hepatosplenomegaly, lymphadenopathy, hypoproteinemia associated with hypereosinophilia, elevated serum IgE, and presence of activated, anergic T cells. The immunological features of four of them have been previously reported (Chilosi et al., 1996; Brugnoni et al., 1997), while the other three are described in Experimental Procedures. The 7 OS patients were analyzed for mutations in either the *Rag-1* or *Rag-2* genes. The results obtained at the nucleotide level are summarized in Table 1. Six of the patients (OS2–OS7) were found to have an abnormality in the *Rag-1* gene. Two patients were homozygous for a missense mutation, one (OS2) for a G1794A mutation causing an R561H change, the other (OS6) for a C1298T transition leading to an R396C substitution. The four remaining patients were compound heterozygotes bearing missense mutations or deletions. Patient OS5 had a 2 nt deletion (nt 368–369) in the first allele leading to frameshift and premature translational termination and an A1398G transition causing a D429G substitution in the second allele. Patient OS4 had a deletion of 13 nucleotides (nt 1723–1735) in the first allele resulting in frameshift and premature termination and a G1299A transition affecting the same codon of patient OS6, but leading to a different amino acid substitution (R396H). Patient OS3 had two missense mutations, the first (R396C) is identical to the one found in patient OS6, while the second (A2847G) is found near the C terminus of Rag-1 (Y912C). Finally, patient OS7 also had two missense mutations, the first affecting a different nucleotide (C1793T) in the same codon as patient OS2 and causing an R561C change, the second a G2322A transition, which causes an R738H substitution.

Residues R396 and D429 are within the homeodomain region of Rag-1 (Figure 1A) (Spanopoulou et al., 1996). R396 represents the fifth residue of the pentapeptide that shows absolute identity between the Hin and Rag-1 DNA-binding domains and has been proposed to mediate binding of the protein to the nonamer motif of the RSS (Difilippantonio et al., 1996; Spanopoulou et al., 1996). D429 is located at the beginning of helix III of the Rag-1 homeodomain (Spanopoulou et al., 1996), while R561 is within the region of Rag-1 that mediates interaction with Rag-2 (McMahan et al., 1997) (Figure 1A). All three amino acids of Rag-1, R396, D429, and R561 have been conserved throughout evolution (Sadofsky et al., 1993).

The seventh patient (OS1) carried two missense mutations in the *Rag-2* gene, a C1324G transversion and a T2055G transversion, causing a C41W and an M285R substitution, respectively. Both mutations are within the Rag-2 active core (Sadofsky et al., 1994; Cuomo and Oettinger, 1994) and involve amino acids conserved in

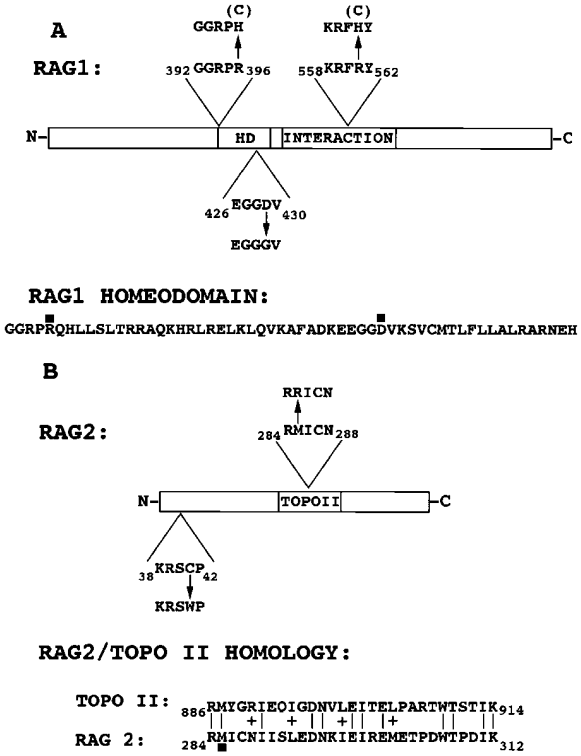

Figure 1. Localization of OS Missense Mutations within the Rag-1 and Rag-2 Proteins

(A) Mutations R396H, R396C, and D429G map within the homeodomain (HD) region of Rag-1 (Spanopoulou et al., 1996). Mutations R561H and R561C map within the Rag-1 domain of interaction with Rag-2 (McMahan et al., 1997).

(B) Rag-2 OS mutations C41W and M285R. The latter localizes within the topoisomerase II homologous region of Rag-2 (Silver, 1994).

all the Rag-2 proteins from mammals, birds, and fish (Sadofsky et al., 1994; Bernstein et al., 1996). M285 maps within a domain of Rag-2 demonstrating high homology to the nonspecific DNA-binding domain of topoisomerase II (Figure 1B) (Silver et al., 1993; Berger et al., 1996).

Mutations of patients OS1 through OS4, for whom parent DNA was available, were traced back to their parents. All parents were heterozygous for the *Rag* mutant alleles and had normal counts of B and T cells. The OS phenotype correlated with the presence of two mutant *Rag-1* or *Rag-2* alleles in their offspring. The possibility that the missense mutations represent rare polymorphisms was excluded by analysis of ethnically matched populations. Rag-1 A2847G and Rag-2 C1324G and T2055G point mutations were from Italian patients and, as they modify a restriction enzyme site, they were analyzed by RFLP analysis of amplified PCR products, in 260 chromosomes from Italian control individuals. The remaining missense mutations were found in Italian (G1794A, C1298T, G1299A), American (C1298T, C1793T, G2322A), and in one case in a Yugoslavian (A1398G) patient. We therefore sequenced 100 chromosomes from American controls; in addition, we performed SSCP analysis on 100 chromosomes from Italian controls. In no case were any of these mutations detected.

Analysis of the TCRβ Repertoire of OS Patients

All seven patients had variable yet detectable numbers of T cells. To investigate the effect of the OS mutations on V(D)J recombination, we determined the TCRβ (TCRB) repertoire. Our study included a global analysis of the variable β (TCRBV) usage and the characterization of TCRBV clonality of individual TCRBV segments, as described elsewhere (Bettinardi et al., 1992; Sottini et al., 1996; Imberti et al., 1997; see also Experimental Procedures). In the OS patients studied (OS1 through OS4), the recombination machinery was sufficiently functional to recombine most TCRBV segments. However, the usage of individual TCRBV segments in OS samples differed from that of unrelated healthy infants of the same ethnic origin with the four OS patients (i.e., Italian) (Figure 2, last panel). In addition, TCRBV rearrangements in OS patients showed restricted heterogeneity, as indicated by heteroduplex analysis of several TCRBV subfamilies prepared from patients OS1 to OS4 (Figure 2, first four panels).

To confirm further the restricted heterogeneity of the TCR repertoire in OS, we cloned and sequenced both the TCRBV segments dominantly expressed in OS (such as TCRBV6 in patient OS1, and TCRBV15 in patient OS4), as well as the least represented OS TCRBV segments (such as TCRBV1 segments) (Table 2). This analysis revealed a predominance of one or a few sequences within each of the TCRBV transcripts analyzed in OS patients (Table 2). In contrast, sequencing of 2 different TCRBV transcripts from a control infant demonstrated that 60 out of 61, and 63 out of 64 clones, respectively, were unique. Similar results were obtained with unfractionated lymphocytes from normal adults (Quirós Roldan et al., 1995). In addition, N nucleotides were present in all sequenced junctions suggesting that these patients do not have deficiencies in terminal deoxynucleotidyl transferase (TdT) activity (Table 2).

Functional Analysis of the OS Rag-1 and Rag-2 Mutations

To determine whether the identified Rag mutations in the OS patients can explain the partial immunodeficiency in OS, we tested the OS mutant Rag proteins for their ability to mediate Rag-1/Rag-2 interaction, specific binding of the complex to the RSS, their cleavage activity and, finally, their potential to mediate recombination of an extrachromosomal recombination substrate.

Effect of OS Mutations on the Rag-1/Rag-2 Interaction

Rag-1/Rag-2 interaction was assayed by coexpression of the wild-type or mutant proteins in 293T cells as previously described (Spanopoulou et al., 1995). Rag-1 OS mutations R396H, D429G, and R561H gst fusion proteins (gst-Rag-1) were transiently expressed along with influenza haemaglutinin (HA)-tagged wild-type Rag-2 (Flu-Rag-2) in 293T cells. Lysed cellular extracts were subjected to affinity precipitation using glutathione beads, and the purified complexes were analyzed by Western blot (Figure 3). Rag-1 homeodomain mutants R396H and D429G maintained efficient interaction with Rag-2, while interaction of Rag-1/R561H with Rag-2 was

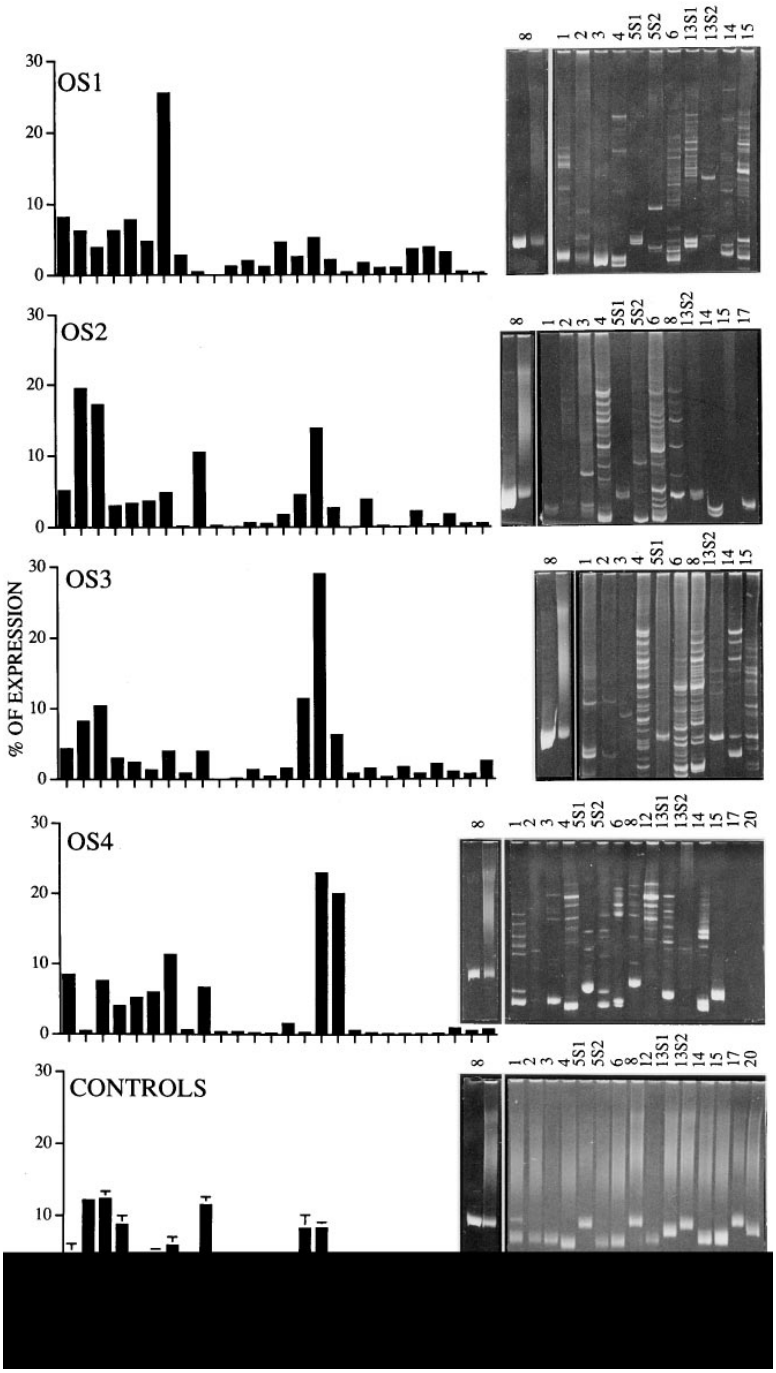

Figure 2. Analysis of the TCRB Repertoire of Total Lymphocytes from OS Patients

(Left) The 4 top graphs indicate the TCRBV usage of peripheral lymphocytes prepared from patients OS1–OS4, while the bottom one represents the mean and standard deviation obtained by analyzing lymphocytes prepared from four unrelated healthy children.

(Right) Heteroduplex analysis of the indicated TCRBV chains obtained by PCR performed with BV family-specific primers on lymphocytes prepared from four OS patients (four top pictures) and from a representative healthy child (bottom). TCRBV8 amplified products, prepared from monoclonal and polyclonal cell preparations, were used as controls and loaded into the first and second lines of each gel.

reduced to about 40% of the wild-type levels (Figure 3A). To test the efficiency of interaction of OS Rag-2 mutants C41W and M285R with Rag-1, gst-Rag-2 proteins were coexpressed with HA-tagged Rag-1 wild-type protein (Figure 3B). Both OS Rag-2 mutations showed reduced interaction with Rag-1 despite the fact that the two mutations are in different parts of the Rag-2 molecule. In all cases, the expression of each individual mutant protein was tested by Western blot analysis of the total cell extract, which revealed that all proteins were expressed at equal levels (Figures 3A and 3B, lower panels).

DNA Binding Activity of the OS Mutants

The effect of OS mutations on the binding of Rag-1/Rag-2 to the RSS was tested by electrophoretic mobility shift assays (EMSAs) (Hiom and Gellert, 1997) using purified recombinant proteins (Spanopoulou et al., 1996). Binding of wild-type Rag-1/Rag-2 to the RSS produces two complexes (Figure 4, lane 1) that both contain Rag-1 and Rag-2 (Santagata et al., 1998). The upper complex results from homodimerization of Rag-1, in part through its homeodomain region (V. Aidinis and E. S., unpublished data), while the lower complex represents the monomeric form of the Rag-1/Rag-2 heterodimer. Muta-

Table 2. Sequences of TCRB Transcript from Omenn Syndrome (OS) Patients

	TCRBV	N[a]	TCRBD[b]	N[a]	TCRBJ[d]		Frequency
OS4	TCRBV1						
	CTGTGCCA	CC	GGACAGGGGG	GAGGG	.....ACTGAAG	(1S1)	9/14
	CTGTGCCAGCAG	GTC	GACAGGG	AAAA	.......ATACGC	(2S3)	3/14
	CTGTGCCAGCAGCGTAG		GGAGG	ATAGC	.......GACCC	(2S5)	2/14
	TCRBV15						
	CTGTGCCACCAGTGATT . .	CATACATCC	GGGACAGGG		.GCACAGATACGC	(2S3)	13/13
OS1	TCRBV1						
	CTGTGCCAGCA		GACAGGGG		.......AATTCACCCC	(1S6)	14/28
	CTGTGCCAGCAGCGTAG		GCG	TC	.....GAGACCC	(2S5)	3/28
	CTGTGCCAGCAGCGTAG		GGGG		......CGAGC	(2S7)	1/28
	CTGTGCCAGCAGCGTAG		GGACTAG		...CTACGAGC	(2S7)	1/28
	CTGTGCCAGCAGCGTAG	A	AGGGGGC	C	..CCTACGAGC	(2S7)	6/28
	CTGTGCCAGCAGC	CC	GGGG	AA	CTCCTACGAGC	(2S7)	1/28
	CTGTGCCAGCAGC	CCGG	CAGGG	CCTG	..CCTACGAGC	(2S7)	1/28
	CTGTGCCAGCAGC	CCCGCCGTAAG[c]			CTCCTACGAGC	(2S7)	1/28
	TCRBV6						
	CTGTGCCAGCA	CCCGATTGATC	GGGGC	CCCACAAGT	..CACAGATACGC	(2S3)	1/10
	CTGTGCCAGCAGCT		GGACAGGGGG		...CTACGAGC	(2S7)	8/10
	CTGTGCCAGCAGCTTA . .	ATTGG	TAGCG	AGGG	......CGAGC	(2S7)	1/10

[a] A few of these nucleotides can be considered as P nucleotides.
[b] The underlined sequences pertain to D1, while the others are from D2.
[c] Two of these nucleotides could be derived from the D sequence.
[d] The specific J segments are indicated in parentheses.

tion R396H decreased the affinity of both complexes (Figure 4, lane 2). The second OS mutation in the Rag-1 homeodomain (D429G) produced a different result. This mutation had little effect on the binding of the lower Rag-1/Rag-2 complex to the RSS but it severely decreased formation of the upper complex (Figure 4, lane 3). Thus, D429G specifically disrupts Rag-1 homodimerization. The third Rag-1 mutation, R561H decreased binding of both the upper and the lower complexes (Figure 4, lane 4). The decreased DNA binding could be due to the lower efficiency of Rag-1 R561H/Rag-2 interaction (Figure 3A). Rag-2 mutants C41W and M285R gave no detectable DNA binding activity (Figure 4, lanes 5 and 6), which could be partly accounted for by their low efficiency of interaction with Rag-1 (Figure 3).

Effect of OS Mutations on the Rag-1/Rag-2 Cleavage Activity

The cleavage activity of the Rag-1 and Rag-2 OS mutants was tested by incubation of purified recombinant Rag proteins with a 12RSS substrate labeled at the 5′ end of the top strand. Conditions for the in vitro cleavage reactions were similar to the DNA binding conditions described by Hiom and Gellert (1997) (see Experimental Procedures and Santagata et al., 1998). The nicking activity of all Rag-1 and Rag-2 OS mutants was directly proportional to their DNA binding activity (compare Figures 4 and 5), while hairpin formation followed a slower rate. Rag-1 R396H had cleavage activity at about 30% of that of the wild-type protein (Figure 5A, lanes 2 and 6) (similar activity was observed for DNA binding, see Figure 3). Rag-1 D429G shows approximately 30% of the wild-type activity (Figure 5A, lanes 3 and 7), while R561H retains only 10% of the wild-type activity (Figure 5A, lanes 4 and 8). OS mutations C41W and M285R in Rag-2 showed no obvious cleavage in accordance with their diminished DNA binding activity (Figure 5B).

Recombination Activity of OS Mutants on Extrachromosomal Substrates

To test the ability of OS mutants to mediate recombination of an extrachromosomal substrate in vivo. Rag-1, Rag-2, and the recombination substrates pJH200 (deletional) and pJH288 (inversional) (Lieber et al., 1988) were cotransfected in NIH 3T3 cells. Recombination products were determined either by PCR analysis (Roman and Baltimore 1996; Spanopoulou et al., 1996) or by Cam^R.Amp^R versus Amp^R (Table 3). OS mutations within the Rag-1 homeodomain drastically reduced the ability of the mutant proteins to mediate recombination of the two substrates (Figure 6, lanes 3 and 4; Table 3). R396H produced only 5%–10% of the wild-type recombination activity whereas D429G impaired recombination on the pJH288 substrate and produced 20% of the wild-type activity on the pJH200 substrate (Figure 6A, lanes 3 and 4; Table 3). The Rag-1 R561H OS mutation that decreases efficiency of Rag-1/Rag-2 interaction reduced overall recombination efficiency to approximately 1/3 of that of the wild-type protein (Figure 6A lane 5; Table 3). All three OS Rag-1 mutations exhibited a similar phenotype when analyzed either in the context of the active core or as full-length proteins, for in vivo recombination (data not shown).

The two OS mutations in Rag-2 showed a differential phenotype. C41W showed diminished recombination activity (Figure 6B, lane 8), in accordance with its DNA binding and cleavage activities (see Figures 4 and 5). However, M285R was able to mediate recombination of the two recombination substrates at 15%–20% of the wild-type levels (Figure 6B, lane 9). Overall, all OS mutant proteins exhibited lower recombination efficiency in mediating inversion of the pJH288 substrate than deletion of the pJH200 substrate (Figure 6). An aliquot of the cells used for the recombination assays was lysed and probed by Western analysis for expression of the various recombinant Rag proteins (Figure 6, lower panel).

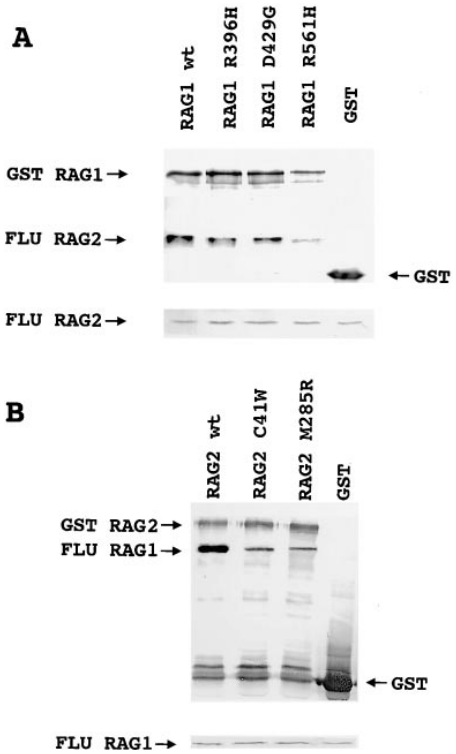

Figure 3. Effect of OS Mutations on the Rag-1/Rag-2 Interaction

Wild-type or mutant proteins were transiently expressed in 293T cells and interaction was evaluated by coprecipitation assays.

(A) Rag-1 OS mutants R396H, D429G and R561H were expressed as gst fusion proteins in the context of the human Rag-1 protein active core (aa 333–1043). Recombinant Rag-1 proteins were coexpressed with HA-tagged Rag-2 active core (aa 1–388).

(B) Rag-2 OS mutants C41W and M285R were expressed as gst fusion proteins in the context of full-length human Rag-2. Recombinant proteins were coexpressed with HA-tagged Rag-1 active core. The lower panels represent Western analysis of the total cell lysates to detect the levels of expression of each recombinant protein.

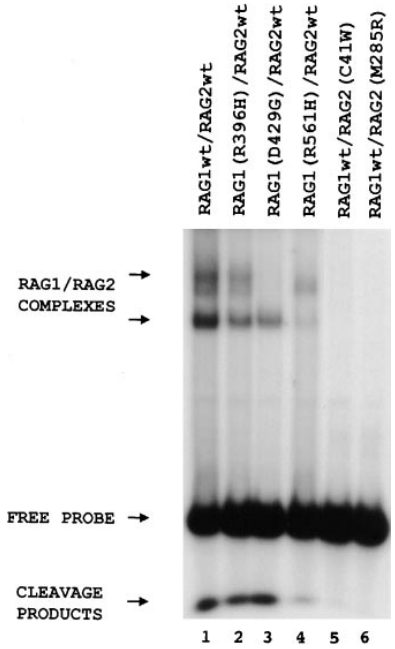

Figure 4. DNA Binding Profile of OS Mutant Rag-1 and Rag-2 Proteins

Purified proteins were incubated with ^{32}P-labeled oligonucleotide containing the 12RSS heptamer-nonamer motifs; ternary complexes were cross-linked with glutaraldehyde and resolved by EMSA as previously described (Hiom and Gellert, 1997). Cleavage products can be visualized at the bottom of the gel.

Discussion

We present evidence that a defect in the *Rag-1* and *Rag-2* genes is responsible for Omenn syndrome, a severe combined immunodeficiency with substantial numbers of activated, oligoclonal T cells. This finding is surprising because previously described Rag mutations led to a complete absence of mature B and T cells, as manifested by the SCID phenotype in both humans and mice (Mombaerts et al., 1992; Shinkai et al., 1992; Schwarz et al., 1996). Three factors demonstrate the link between mutations in the Rag genes and the appearance of the OS phenotype. First, appearance of the OS immunodeficiency correlates with mutations on both *Rag-1* or *Rag-2* chromosomal alleles. Second, analysis of more than 400 chromosomes from healthy individuals, ethnically matched to the OS patients, showed that these mutations do not represent polymorphisms in the general population. Third, all Rag mutations detected in OS patients drastically reduce V(D)J recombination as judged by in vitro and in vivo assays. From the genetic and biochemical data reported in this paper, it can be concluded that the defect in Omenn syndrome is caused by mutations in the *Rag-1* and *Rag-2* genes that confer partial activity and allow only a low degree of V(D)J recombination to occur.

Genotype of OS

Five of the OS missense alleles affect either the 396 or 561 Rag-1 codons, but in four of the cases different nucleotides are involved. This finding, together with the fact that the two patients with the same C1298T transition are of different ethnic origin (OS6 is English-American, while OS3 is Italian), suggests that the mutated codons could represent a hot spot for mutations. An alternative explanation, however, would be that only a limited number of residues in the Rag genes can be altered in order to maintain the partial recombinase activity necessary for the OS phenotype. Previous analyses of B^-T^- SCID patients have shown that the complete lack of mature B and T cells in those patients resulted from either truncations of the Rag genes or missense mutations in the active core of the two proteins that eliminated Rag function (Schwarz et al., 1996). In contrast, the OS mutant Rag proteins retain part of their activity, which appears to account for the recombination events present in the T cells of these patients. The issue then arises as to what level of Rag activity is required for the establishment of a functional immune system. The fact that the parents that are heterozygotes for *Rag-1* deletions 1723–1735 and 368–369 represent healthy individuals with normal counts of B and T cells, suggests that expression of only one allele is sufficient

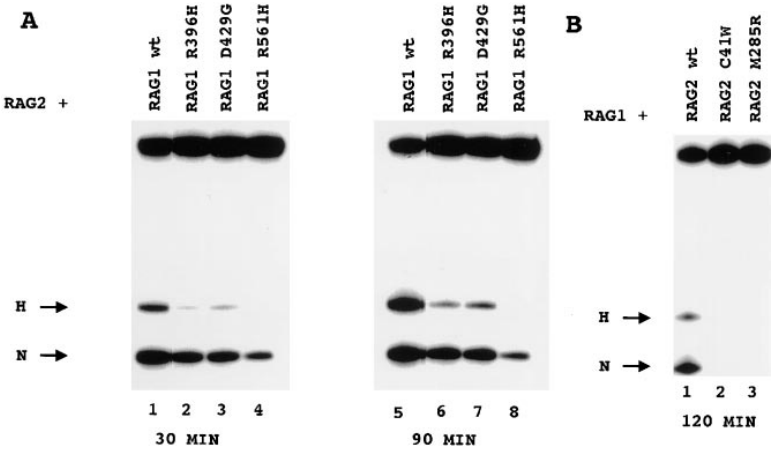

Figure 5. Cleavage Activity of OS Mutant Rag-1 and Rag-2 Proteins

Purified proteins were incubated with the 12RSS substrate labeled on the upper strand, and cleavage products, nicked intermediates, and covalently sealed hairpins were resolved on a denaturing polyacrylamide gel.

for normal V(D)J recombination and immunocompetence. In addition, none of the OS missense mutations have a dominant negative effect since the heterozygous parents have a normal immune system.

OS Mutations that Affect Rag-1/Rag-2 Interaction

Three of the OS mutations, Rag-1 R561H, Rag-2 C41W, and Rag-2 M285R, have a significant effect on the Rag-1/Rag-2 interaction. R561H is within a region of Rag-1 that mediates interaction with Rag-2 (McMahan et al., 1997) and decreases efficiency of interaction by ~80%. Mutations that affect the Rag-1/Rag-2 association might have a more dramatic effect during recombination in lymphocytes than when assayed in the in vitro or fibroblast assays. In lymphocytes, endogenous Rag-1 and Rag-2 are present in limiting amounts, and efficiency of Rag-1/Rag-2 interaction is critical for V(D)J recombination to occur. In contrast, both the in vitro and fibroblast assays are conducted in the presence of high levels of Rag-1 and Rag-2 augmenting the efficiency of their interaction. It is therefore possible that the effect of Rag-1 R561 in lymphocytes is far more pronounced than in the in vitro assays.

OS mutation C41W in Rag-2 provides a radical change by the substitution of a Cys residue with Trp. This mutation shows diminished DNA binding and cleavage activity that might result from a global change of the tertiary structure of the protein. The other OS Rag-2 mutation, M285R, maps within a region of Rag-2 that shows distinct homology to the nonspecific DNA-binding domain of topoisomerase II (Silver, 1994; Berger et al., 1996). The function of this topo II homologous domain of Rag-2 remains to be defined. M285R follows the pattern of C41W in that it interacts with Rag-1 with lower efficiency and has almost undetectable DNA binding and cleavage activity in vitro. However, in vivo the protein retained about 20% of the wild-type activity. This enhanced in vivo activity might be conferred by conformational changes through the interaction with other cellular proteins.

OS Mutations within the Rag-1 Homeodomain

Three of the seven OS patients characterized in this study carried a missense mutation at position R396. R396 is at the N terminal of the Rag-1 homeodomain within the region of Rag-1 (GGRPR, aa 392–396) that shows absolute homology to the DNA-binding domain of the Hin invertase (Difilippantonio et al., 1996; Spanopoulou et al., 1996). We have previously shown that the equivalent positions in mouse Rag-1 are important for binding of the protein to the nonamer motif (Spanopoulou et al., 1996). Mutation of R396 in the human gene reduces binding of the protein to the RSS, while it has no effect on the Rag-1/Rag-2 interaction. Given the effect of this mutation on the repertoire of the OS patients the

Table 3. In Vivo Recombination Activity of OS Mutants

Amino Acid Mutation	PCR Relative Recomb.		pJH288		
	pJH288	pJH200	$Cam^R.Amp^R/Amp^R$	Efficiency	Relative Recomb.
Rag-1 WT	100%	100%	232/26,710	0.86%	100%
Rag-1 R396H	5%	10%	39/28,230	0.14%	16%
Rag-1 #396C	NA	NA	NA	—	—
Rag-1 D429G	5%	20%	18/25,370	0.07%	8%
Rag-1 R561H	23%	34%	63/27,300	0.23%	27%
Rag-1 R561C	NA	NA	NA	—	—
Rag-1 R737H	8%	16%	15/29,840	0.05%	6%
Rag-1 Y912C	NA	NA	NA	—	—
Rag-2 C41W	4%	10%	12/19,740	0.06%	7%
Rag-2 M285R	15%	30%	46/25,680	0.18%	22%

Values represent the average of five independent experiments analyzed by PCR and two analyzed by bacterial transformation. Statistical variation for recombination efficiency by bacterial transformation ranged from 0.01–0.03% depending on the sample.
NA; not analyzed.

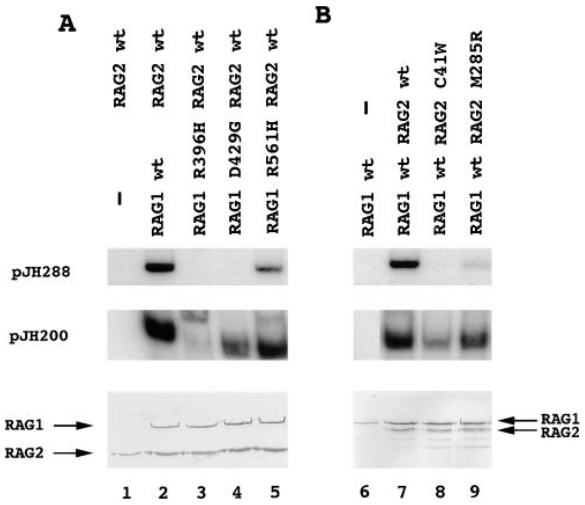

Figure 6. Effect of OS Mutations on V(D)J Recombination Activity

(A) OS human Rag-1 mutants were expressed in the context of the active core protein. Human Rag-2 mutants were expressed as full length form. OS mutant Rag-1 or Rag-2 were expressed from the peBG cassette (Spanopoulou et al., 1996). Plasmids expressing the recombinant proteins were cotransfected along with the recombination substrates pJH200 or pJH288 (Lieber et al., 1988) in NIH 3T3 cells, and 48 hr later recombination products were isolated. Recombination efficiency was detected by PCR analysis using primers that detect the recombined sequences (Roman and Baltimore, 1996). To control for transfection efficiency and production of the recombinant proteins, an aliquot of the cell lysate was analyzed by Western analysis for the expression of the recombinant proteins (bottom panel) detected with an anti-gst antibody.

question becomes whether low-affinity binding of Rag-1 mutants to the nonamer motif might drive preferential recombination of certain nonamer elements and/or heptamer-only dependent rearrangement. The availability of an animal model for the Rag-1 R396 mutation would provide a tool to analyze the repertoire generated by such a mutation and the role of the Rag-1 homeodomain in vivo.

Mutation OS Rag-1/D429G Affects Rag-1 Homodimerization

The other OS mutation within the Rag-1 homeodomain, D429G, maps to the first residue of the putative homeodomain helix III (Spanopoulou et al., 1996). The mutation specifically eliminates formation of the upper Rag-1/Rag-2 complex, which is partly mediated by homodimerization of the homeodomain (V. Aidinis and E. S., unpublished data). Therefore, D429 contributes to Rag-1 homodimerization, which might be crucial for synaptic complex formation. Homodimerization of homeodomain regions has been documented for the *Paired* class of homeodomains. The crystal structure of the *Paired* homeodomain has shown that dimer formation is mediated by a set of amino acids in the interphase of two domains that includes an acidic amino acid, Glu-42, present at the first position of recognition helix III (Wilson et al., 1995). Asp-429 of the Rag-1 homeodomain is present at the equivalent position with Glu-42. In this context, it is interesting to note that mutation of the first residue, E80A, in the recognition helix III of the CRX homeodomain also leads to disease. CRX is a photoreceptor-specific transcription factor that belongs to the *Paired* class of homedomains. Mutation of Glu-80 to Ala leads to cone-rod dystrophy in humans (Freund et al., 1997).

Phenotype of OS

A major question that remains to be answered is why partial V(D)J recombination activity leads to the OS phenotype. OS immunodeficiency is characterized by an overrepresentation of Th2-type cells (as judged by increased secretion of IL-4, IL-5, and IL-10), elevated serum IgE, and eosinophilia (Schandené et al., 1993; Chilosi et al., 1996; Brugnoni et al., 1997). The genetic defect in OS allows productive rearrangement of multiple TCRBV segments, yet the TCR repertoire in peripheral blood T cells is mostly oligoclonal. Selective expansion of TCR clones may be a consequence of either intrathymic selection of specific rearrangements or peripheral expansion in response to infections, or both. In addition, it is possible that mutations in the nonamer DNA-binding domain of Rag-1 could lead to preferential recombination of certain TCRBV segments. Skewing toward Th2 might be the result rather than the cause of the OS phenotype. As observed in other cases, immunedeficient individuals have, by definition, an increased antigen exposure and a defect in antigen clearance that results in persistent high antigen load. If, as in OS, the genetic defect is permissive and allows the development of limited clones of mature T cells, the antigen overload favors prolonged T cell activation that has been associated with increased IL-4 secretion and polarization toward a Th2 phenotype (Hsieh et al., 1993; Hosken et al., 1995).

The high IgE levels and eosinophilia observed in OS patients can be a direct response to the selective expansion of Th2 clones. In several mouse models high levels of IgE production have been associated with the expansion of the NK1.1, IL-4 producing, Th2 cells (Yoshimoto et al., 1995; Bendelac et al., 1996). In addition, elevated serum IgE has been associated with the absence of the TCR$\alpha\beta$ lineage in TCRα-deficient mice (Wen et al., 1994). In all of the above cases, as in OS, there is an imbalance in the T–B network of interactions due to the preferential expansion of certain T cell subsets with consequent altered secretion of particular cytokines and Ig isotypes. This imbalance must be characteristic of only some lymphoid abnormalities. Other immunodeficient organisms, such as the V(D)J recombination-deficient SCID mouse that has low levels of T cells or genetically engineered mice with low numbers of B cells (such as the $\lambda 5^{-/-}$ mice), do not manifest a Th2 phenotype or high levels of IgE. Definitive answers on the pathogenesis of OS will come from the establishment of animal models for the different OS mutations.

V(D)J Recombination Activity in Omenn Syndrome

The data present in this study show that the immunodeficiency in OS arises from missense mutations in the Rag proteins that confer partial V(D)J recombination activity. All of the OS patients (7/7) analyzed in this study carried mutations in the Rag genes that can account for the

"leakiness" of the OS phenotype. However, it is possible that certain mutations in the ubiquitous components of the V(D)J recombination machinery could also lead to an Omenn-like phenotype. For example, in light of the findings that *Ku70*$^{-/-}$ mice have low levels of T cells and develop T cell lymphomas (Gu et al., 1997), it is conceivable that a partial Ku70 function could produce an OS phenotype associated with T cell lymphomas (Mache et al., 1994). V(D)J recombination deficiencies that lead to low-to-normal numbers of T cells in the absence of mature B cells are a recurring theme. When the differentiation defect in *Rag*$^{-/-}$ or SCID mice is rescued by genetic manipulation such as p53 or poly(ADP-ribose) polymerase (PARP) inactivation or by damaging agents, the T cell, but not the B cell compartment, is restored (Danska et al., 1994; Guidos et al., 1995, 1996; Bogue and Roth, 1996; Livak et al., 1996; Nacht et al., 1996; Morrison et al., 1997). The mechanisms underlying these differences between B and T cell development remain to be defined. The elucidation of the molecular basis of the defect in Omenn syndrome will permit the investigation of these mechanisms as well as the clinical features of the disease.

Experimental Procedures

Patients

We studied seven patients with typical signs of OS. The immunological features of four patients have been previously reported. Patient OS1 corresponds to G. M. in Chilosi et al., 1996. Patients OS2, OS3, and OS4 correspond to R. C., C. A., and C.N., respectively, in Brugnoni et al., 1997. Patient OS5, the female child of non consanguineous parents, developed generalized erythrodermia in the neonatal period and chronic diarrhea and failure to thrive at two months of age. At that time, eosinophilia (2024 cells/mm3) and increased serum IgE (500 U/ml) were manifested. Lymphocyte subpopulations (%) were as follows: CD3 = 11, CD19 < 1, CD16 = 57. Virtually all $CD3^+$ cells coexpressed CD45R0, a marker for primed T cells, and 93% of them were also DR^+. In vitro lymphocyte proliferative responses to phytohemagglutinin (PHA) and anti-CD3 monoclonal antibody were abolished. Patient OS6 had eosinophilia (1400/mm^3), normal numbers of peripheral blood lymphocytes (2,750/mm^3), the majority (88%) being $CD16^+$ NK cells, with severely reduced $CD3^+$ cells (4%), and absence of B cells. Virtually all circulating T lymphocytes coexpressed the DR activation antigen. In vitro lymphocyte proliferation to PHA was abolished.

Patient OS7 developed generalized erythrodermia associated with lymphoadenopathy at 3 weeks of age and, subsequently, presented with diarrhea, failure to thrive, splenomegaly, pneumonia, anemia, thrombocytopenia, and eosinophilia (5,200 cells/mm^3). She had elevated serum IgE (800 U/ml at 3 months of age and 45,000 U/ml at 5 months) and an increased lymphocyte count (5,000–13,000/mm^3), with 54% $CD3^+$ T cells, most of which (74%) coexpressed the DR antigen, and 2% B cells. The in vitro proliferative response to PHA was markedly reduced. Patients OS5 and OS7 have died after unsuccessful bone marrow transplantation, and sepsis, respectively. All other patients have been cured by bone marrow transplantation. In all patients, the autologous origin of T lymphocytes (to rule out maternal T cell engraftment) was demonstrated by HLA typing and by molecular analysis with highly polymorphic markers (D1S80, APOB).

Strategy for Mutation Identification in the *Rag-1* and *Rag-2* Genes

Rag-1 and *Rag-2* coding sequences were amplified from genomic DNA. All the patient DNAs were obtained before bone marrow transplantation. DNAs were obtained also from the parents of OS1, OS2, OS3, and OS4 patients. Primers were designed for the amplification of the Rag genes based on the sequences reported in databases (*Rag-1*: a.n. M29474; *Rag-2*: a.n. M94633). *Rag-1* gene was amplified in two segments (94–1852 and 1781–3262) and *Rag-2* in one segment (1201–2922). Sequencing was performed directly on the PCR products purified from the gel with the Thermosequenase kit (Amersham, U. K.). Mutations were confirmed by analysis of several clones from PCR amplification products cloned in TA vector (Invitrogen) and sequenced by the dideoxynucleotide chain termination method using the Sequenase kit (USB), as previously described (Macchi et al., 1995). For the analysis of restriction enzyme sites eliminated or created by the mutations, the pertinent PCR products were digested with the appropriate enzyme according to the suggestions of the manufacturer. Single-stranded chain polymorphism (SSCP) was performed on small PCR products amplified with pertinent primers and run according to standard methods.

Analysis of TCRB Repertoire

Total cytoplasmic RNA and cDNA were prepared from peripheral blood lymphocytes as previously described (Bettinardi et al., 1992), and the cDNA was subjected to enzymatic amplification using a TCRBC primer (βAI: 5′ CCC ACT GTG CAC CTC CTT CC 3′) and a TCRBV degenerated primer: Vβd: 5′ ACG TGA ATT CT(GT) T(ACT) (CT) TGG TA(CT) (AC)(AG)(AT) CA 3′. This amplification generates a product of about 360 bp, which contains one-half of the V region gene and extends through the VDJ junctions until the first 165 bp of the C region. The 40 cycles of PCR were carried out by combining 1 μl of cDNA in 100 μl of reaction volume containing 2.5 U of Taq polymerase, dNTP at the final concentration of 250 μM each and 150 pmol of the Vβd primer and 50 pmol of the βAI primer in a buffer prepared with 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, and 100 μg/ml of BSA. The PCR conditions were as previously described (Imberti et al., 1997).

The specificity of the total amplified products was analyzed using a colorimetric method (Bettinardi et al., 1992) and biotinylated TCRBV-specific probes. The relative percentage of expression of each individual TCRBV segment was calculated by dividing the single TCRBV signals by the sum of the signals for all TCRBV families and multiplying by 100. Subsequently, the TCRBV of interest were individually amplified by 35 cycles of PCR, using TCRBV-specific oligonucleotides and the βAI TCRBC primer, and subjected to heteroduplex analysis as previously described (Sottini et al., 1996). Samples were denaturated at 95°C for 5 min and then kept at 50°C, a temperature that is permissive for the annealing between either homologous (homoduplex) or heterologous DNA strands, which have the same TCRBV family but differ for the N and the TCRBJ regions (heteroduplex). The annealed samples were run on a 12% acrylamide gel, and the gels were stained with ethidium bromide and photographed under UV light. Amplified TRCBV8 products from the T cell line J77 and from lymphocytes stimulated with an anti-TCRBV8 monoclonal antibody were used as monoclonal and polyclonal controls, respectively (Sottini et al., 1996). Specific PCR products of interest were purified, cloned, and sequenced as described (Sottini et al., 1996). The sequences were compared with published data relative to TCRBV, TCRBD, TCRBJ, and TCRBC segments (Arden et al., 1995).

Constructs

PCR products containing the mutations found in OS patients were cloned in TA vector and sequenced. Subsequently, PCR products were cloned in pEBG according to the general strategy of substituting restriction fragments cut with pertinent enzymes to the wild-type genes cloned in this vector. For protein expression, aa 330–1044 of human Rag-1 (Rag-1 active core; Sadofsky et al., 1993; Silver et al., 1993) and aa 1–527 of human Rag-2 or the corresponding fragments from the mutant proteins were subcloned into the mammalian expression vector pEBG, which provides the coding sequences for the glutathione transferase gene (*gst*) (Spanopoulou et al., 1995).

Protein Expression and Purification

Gst-fusion recombinant forms of the human Rag-1 and Rag-2 proteins were overexpressed in 293T cells and purified as previously described (Spanopoulou et al., 1996). Proteins were dialyzed in cleavage buffer and quantified by Coomassie blue staining following SDS–PAGE.

In Vitro Cleavage Reactions

Standard reaction conditions were modifications of previously published protocols (McBlane et al., 1995; Hiom and Gellert, 1997; Santagata et al., 1998). Fifty nanograms of each protein, Rag-1 and Rag-2, was incubated with 0.01–0.05 pmol of ^{32}P end-labeled cleavage substrate in 25 mM MOPS-KOH (pH 7.0), 5 mM Tris-HCl (pH 8.0), 120 mM KOAc, 18 mM KCl, 10% dimethyl sulfoxide (DMSO), 2.2 mM DTT, 1mM Mg^{2+}, 0.5 mM nonspecific single-stranded DNA with 50 ng of each protein and 0.01–0.05 pmol of ^{32}P end-labeled probe in a final volume of 20 μl. Reactions were stopped by the addition of 0.1% SDS and loading buffer. Samples were analyzed on 16% polyacrylamide denaturing gels. The upper strand sequence of 12RSS cleavage substrate is: 5′-ACGCGTCGACGTCTACACAG TGATAAGCCCTGAACAAAAACCGGATCCGCG-3′. Standard cleavage reactions employed ^{32}P 5′ end-labeled upper strand annealed to the unlabeled, complementary lower strand oligonucleotide.

Electrophoretic Mobility Shift Assays

Conditions were based on the previously published protocol (Hiom and Gellert, 1997). Fifty nanograms of each protein was incubated for 10 min at 30°C with 0.01–0.05 pmol of 5′ end-labeled probe in 25 mM MOPS-HCl (pH 7.0), 1 mM $MgCl_2$, 2.2 mM DTT, 1 μg of bovine serum albumin (BSA), 0.5 μM of nonspecific primer (5′-CCTC GAGCTCA TCAGCTTGCCTGTGGGCAGCTCGATCTCTTTGTCG-3′), 20% DMSO, and 120 mM potassium acetate. Cross-linking was achieved by glutaraldehyde (final concentration 0.1% v/v) treatment for 10 min at 30°C. Complexes were resolved on a 4% native polyacrylamide gel.

Rag-1/Rag-2 Protein Interaction Assays

Recombinant proteins, human gst-Rag-1 wild type or OS mutants and mouse influenza HA-tagged Rag-2 (Flu-Rag-2) or mouse HA-tagged active core Rag-1 (Flu-Rag-1) and human gst-Rag-2, were transiently transfected in 293T cells and after 48 hr their interaction potential was assessed by coprecipitation assays as previously described (Spanopoulou et al., 1995).

In Vivo Recombination Assays

Mutant proteins were tested for their recombination activity in NIH 3T3 cells. Human Rag-1 active core (aa 330–1043) was cotransfected with mouse wild-type Rag-2 active core (aa 1–388) whereas human full-length Rag-2 proteins were cotransfected with mouse Rag-1 active core (aa 330–1040). Recombinant constructs were expressed in NIH 3T3 cells along with the recombination substrates pJH288 or pJH200 (Lieber et al., 1988). Cells were transfected by calcium phosphate precipitation and harvested 48 hr later. Recombined products were isolated as described previously (Oettinger et al., 1990) and analyzed for recombination frequency by PCR analysis (Roman and Baltimore, 1996). To ensure that the PCR assays follow a quantitative pattern, reactions were allowed to proceed for 25 cycles and controlled by limited dilutions (1/2, 1/10, 1/100, 1/1000) of a recombined pJH288 plasmid DNA. Oligonucleotides detect the recombined products by annealing to the joined heptamer signal sequences (oligo-RA5) and to the *CAT* gene (oligo-RA14) (RA5: 5′-CCAGTCTGTAGCACTGTGCAC-3′ and RA14: 5′-TCCAGCTGAA CGGTCTGGT-3′). The reactions incorporated [^{32}P]dCTP. Reaction products were analyzed on a 5% polyacrylamide gel and visualized by autoradiography. Relative recombination activity was estimated by phosphoimaging (Biorad). Alternatively, efficiency of recombination was estimated by transformation of the recovered plasmid DNA into electrocompetent MC1061 bacteria (Biorad) and analysis of the CamR/AmpR versus AmpR ratio (Lieber et al., 1988).

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