Biochemical and Folding Defects in a RAG1 Variant Associated with Omenn Syndrome

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Biochemical and Folding Defects in a RAG1 Variant Associated with Omenn Syndrome

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The RAG1 and RAG2 proteins are required to assemble mature Ag receptor genes in developing lymphocytes. Hypomorphic mutations in the gene encoding RAG1 are associated with Omenn syndrome, a primary immunodeficiency. We explored the biochemical defects resulting from a mutation identified in an Omenn syndrome patient which generates an amino acid substitution in the RAG1 RING finger/ubiquitin ligase domain (C325Y in murine RAG1) as well as an adjacent substitution (P326G). RAG1 C325Y demonstrated a 50-fold reduction in recombination activity in cultured pro-B cells despite the fact that its expression and localization to the nucleus were similar to the wild-type protein. The C325Y substitution severely abrogated ubiquitin ligase activity of the purified RAG1 RING finger domain, and the tertiary structure of the domain was altered. The P326G substitution also abrogated ubiquitin ligase activity but had a less severe effect on protein folding. RAG1 P326G also demonstrated a recombination impairment that was most pronounced when RAG1 levels were limiting. Thus, a correctly folded RAG1 RING finger domain is required for normal V(D)J recombination, and RAG1 ubiquitin ligase activity can contribute when the protein is present at relatively low levels. The Journal of Immunology, 2007, 179: 8332–8340.

Omenn syndrome (OS) is a rare, deadly, primary immunodeficiency characterized by an absence of circulating B lymphocytes but variable levels of oligoclonal T lymphocytes. OS features a specific spectrum of clinical manifestations including erythroderma, lymphadenopathy, and frequent severe infections (1, 2). Mutations in RAG1 and RAG2 and less frequently in the DCLRE1C gene, encoding the Artemis protein, or other loci have been identified in OS patients (3–7). The RAG1 and RAG2 proteins constitute a lymphoid-specific endonuclease required for the development of both B and T lymphocytes (8–11). Together they initiate the somatic recombination of variable (V), diversity (D), and joining (J) gene segments (V(D)J recombination) during the assembly of intact Ag receptor genes (reviewed by Gellert; Ref. 12). The RAG1/2 complex introduces double-strand breaks in the DNA at a pair of recombination signal sequences (13, 14). The recombination signal sequence ends are subsequently joined to form signal joints (SJ), whereas the flanking DNA is processed and joined to form coding joints (CJ) that code for the Ag receptors. Deficiencies in V(D)J recombination are believed to account for the OS phenotype (3).

Many RAG1 alleles associated with OS encode hypomorphic proteins (3, 15, 16). The reduced level of recombination activity appears to be sufficient to support some T cell but not B cell development. In at least one case, an amorphic germline RAG1 mutation was found to be suppressed by secondary somatic mutations in the T cell lineage (17), thus allowing for some T cell development. In other cases, the altered proteins appear to support fairly robust levels of recombination on model substrates (16). These alleles are missing portions of the RAG1 N-terminal region (N-TR), and it has been proposed that this region may be specifically required for rearrangement of the Ig loci (16) or for proper cellular localization (15).

The highly conserved core of RAG1 (aa 384-1008 of 1040 in murine RAG1 (mRAG1)) does not include the N-TR. The core is sufficient to complement RAG2 in recombination of extrachromosomal substrates, although it supports markedly reduced levels of recombination on the IgH locus in cultured cells (18–20). The mRAG1 core can also support development of both B and T cells in the mouse, but total V(D)J recombination is reduced and aberrant recombination is increased (21, 22). Although the majority of the missense mutations in the human RAG1 (hRAG1) gene associated with OS result in amino acid substitutions in the core, at least three have been found in the N-TR (7, 23).

The G1095A mutation, resulting in a C328Y substitution, was identified in a 3-month-old patient presenting with some of the clinical features of OS, including skin rash, pneumonia, lymphadenopathy, and hepatomegaly (7). Although this patient displayed a severe reduction in circulating B lymphocytes (<1% of total lymphocytes), the overall immunological phenotype differed in several respects from the typical OS profile. The total lymphocyte count was reduced to 1.0 × 10^9/μl, compared with an average 7.2 ± 2.0 × 10^9/μl for other OS patients and 4.8 ± 0.35 × 10^9/μl for normal controls. The percentages of CD3+, CD19+, and CD16+ cells were similar to other OS patients, but the absolute numbers were reduced relative to both other OS patients and controls. Thus, there was a 10-fold reduction in absolute numbers of circulating CD3+ T cells, at least a 150-fold reduction in CD19+ B cells, and a more modest 3-fold reduction in circulating CD16+ NK cells.
relative to controls. Finally, this patient did not exhibit eosinophilia, erythodera, or elevated levels of circulating IgE, features normally associated with OS. This profile is consistent with a defect in V(D)J recombination that is manifested to different extents in developing B and T lymphocytes.

hRAG1 C328 is a zinc-coordinating residue in the RING finger domain of the protein, the tertiary structure of which is maintained via coordination of three zinc atoms (24). The RING domain (aa 264–389) is present in all known vertebrate RAG1 proteins, and the positions of the zinc-coordinating residues are absolutely conserved (19). As is the case with many RING domains (25), the isolated mRAG1 RING has been shown to possess ubiquitin ligase activity, promoting the covalent attachment of the ubiquitin protein both to itself and to model targets in trans in reconstituted systems (26, 27). The biological function of this activity is currently under investigation, but ubiquitin conjugation in general is thought to play roles in regulation of virtually every cellular process in eukaryotes (28).

RAG1 is unique among known proteins in combining both endonuclease and ubiquitin ligase activities in a single polypeptide. Mutation or deletion of the RING finger in the context of an otherwise intact mRAG1 protein has been shown to reduce recombination of model substrates in cultured fibroblasts and pro-B cells, although this work was performed before the ubiquitin ligase activity of this domain was known (18–20). The specific effects of the hRAG1 C328Y substitution (corresponding to C325Y in mRAG1) on both ubiquitin ligase activity and V(D)J recombination have not been examined. Here we report that the C325Y substitution in mRAG1 disrupts ubiquitin ligase activity and that mRAG1 C325Y supports only very low levels of recombination of model substrates in pro-B cells. This inability to support recombination could not be explained by changes in protein levels or cellular compartmentalization, but the C325Y substitution did alter the tertiary fold of the RING finger domain. Substitution at an adjacent site within the mRAG1 RING, P326G, also disrupted ubiquitin ligase activity and decreased recombination activity, but in this case the recombination defect could be partially overcome by high levels of RAG1 expression. The data indicate that the inability of hRAG1 C328Y to support B cell development is only partly due to a defect in ubiquitin ligase activity, and that a properly folded RING finger domain is required for normal B cell development in humans.

Materials and Methods

Plasmids and cells

pH548 and pH549 encode full-length murine RAG1 and RAG2, respectively (19); pH200, pH290, and pH299 are recombination substrates in which SJ, CJ, or both, respectively, are left in the plasmid backbone after recombination (29, 30); pJMJ029 encodes mRAG1 aa 218–389 (26), including the RING domain and an upstream lysine/arginine-rich region fused in frame with Xpress epitope and 6 histidine (H6) tags. pJMJ072 encodes mRAG1 fused in frame with C-terminal myc and H6 tags. pJMJ072 encodes mRAG1 fused in frame with Xpress epitope and 6 histidine (H6) tags. pJMJ071 and pJMJ072 were derived for this study from pcDNA4.0/myc/his-c (Invitrogen) and pcDNA3.1/myc/his-A (Invitrogen) according to the manufacturer’s instructions. Anti-lamin B1 antibody (Active Motif) was detected by Western blot of urea-solubilized whole-cell lysates using anti-myc Ab (1/2000, Invitrogen) or anti-RAG1 K20 Ab (1/2000, Santa Cruz Biotechnology). Separation of cells (4×10⁷) by centrifugation and insoluble fractions was performed using the Nuclear Extract Kit from Active Motif according to the manufacturer’s instructions. Antimyc Ab (1/1000, Abcam) and anti-cotton/zinc superoxide dismutase 01 (1/1000, Assay Designs) were used as controls (33, 34). For confocal microscopy, NIH 3T3 cells seeded in chamber slides were transfected with pEGFP-N1, pH200 wild-type, or pH200 C325Y (1 μg) using Lipofectamine reagent (Invitrogen) according to the manufacturer’s recommendations. After 24 h, living cells were visualized using an Olympus BX61 confocal microscope and Fluoview software, version 4.3. Images were taken at ×10 magnification with an ×4 digital zoom.

Protein purification and analysis

Purification of wild-type, C325Y, P326G, and C328S forms of RAG1/2 was fused in frame to an H6 tag was performed by nickel affinity chromatography. Proteins were expressed in Rosetta 2 (DE3) cells (Novagen) transformed with pMJC01 (type or P22 helper phage) which was grown and induced as previously described (26). All steps for purification were conducted at 4°C. Whole-cell pellets were solubilized in urea buffer (20 mM Tris (pH 7.2), 0.5 M NaCl, 10 mM β-ME, 8 M urea) and incubated on ice for 1 h. Lysates were cleared by centrifugation (70,000 × g, 30 min), applied to Ni-NTA (Qiagen) columns and washed with urea buffer plus 60 mM imidazole. Proteins were renatured on the column with a linear, 10–column volume gradient from 0 to 0 M urea and eluted with solubilization buffer without urea plus 0.5 M imidazole. Purified proteins were dialyzed overnight against storage buffer (20 mM Tris (pH 7.4), 0.15 M KCl, 0.05 mM ZnCl₂, 10% glycerol, 1 mM DTT) and stored at –80°C. Protein concentrations were determined using Bio-Rad Protein Assay dye reagent (45 mM, CDC34 as indicated, and ubiquitin tagged with a protein kinase motif (500 μM) in reaction buffer (50 mM Tris (pH 7.4), 0.001% Brij, 2 mM Mg-ATP, 50
mM NaCl, 60 mM KCl, 0.4 mM DTT, 0.02 mM ZnCl₂) and incubated at 37°C for 2 h. Products were separated by denaturing gel electrophoresis and subjected to Western blot analysis.

For partial proteolysis, trypsin (20 ng unless otherwise indicated) was added directly to RAG1Δ218-389(4-5/)H9262g) in storage buffer. Reactions were incubated for 2 h (37°C) and terminated with PMFS (2 mM). Proteolysis products were separated on 18% Tris-glycine gels (Invitrogen) and stained with colloidal blue stain (Invitrogen). In some cases, protein fragments were transferred to polyvinylidene difluoride membrane and subjected to N-terminal sequencing at the National Cancer Institute (Frederick, MD).

For Western blot analysis of digested proteins, fragments were separated on 4-12% Bis-Tris gels (Invitrogen), transferred to nitrocellulose, and probed with rabbit antiserum raised against the mRAG1 peptide VSSKEV-LKKISNC (1/5000; Sigma Genosys).

Quantification
All quantification was performed using a Kodak Image Station 2000 mm and Kodak 1D software, version 3.6.5. Agarose gels stained with ethidium bromide were exposed for 30 s with zero binning. In all cases, quantification is provided as net pixel intensity/10⁴. Average and SD were calculated from three or more independent trials.

Results
Defects in mRAG1 C325Y recombination activity
Full-length mRAG1 with a substitution at the equivalent position to hRAG1 C328Y (mRAG1 C325Y) supported only very low levels of recombination on extrachromosomal substrates. RAG1-negative pro-B cells were transiently transfected with extrachromosomal recombination substrates along with the pJH548 plasmid expressing either wild-type or C325Y mRAG1. These recombination substrates are designed such that recombination will result in expression of chloramphenicol acetyltransferase in bacteria, whereas all plasmids confer ampicillin resistance (29). When chloramphenicol resistance was used to score recombination, we detected neither SJ nor CJ formation supported by mRAG1 C325Y (Table I). Wild-type mRAG1 supported SJ formation at a rate of 2.3% and CJ formation at a rate of 1.5%, both of which are within the normal range for recombination of extrachromosomal substrates in B-lineage progenitor cells (35). We next used a more sensitive assay in which recovered plasmid DNA was subjected to semiquantitative PCR. Two independently isolated C325Y clones were tested, and all transfections were performed in triplicate. The averages and SDs derived from quantification of the SJ band generated in three independent transfections are shown below the gel (Fig. 1A, top). The quantity of recombination product detected in the undiluted mRAG1 C325Y samples was less than that seen with a 50-fold dilution of the wild-type sample. Control amplifications performed on serially diluted DpnI-treated DNA indicated no difference in the amount of the replicated recombination substrate (Fig. 1A, bottom). From this, we conclude that the level of SJ formation on pJH200 supported by mRAG1 C325Y was at least 50-fold lower than that supported by wild-type mRAG1. Similar results were obtained when the wild-type and C325Y mRAG1 proteins were expressed from the pJM071 plasmid (Fig. 1B; top, SJ; bottom, total template), which included a more robust promoter.

Table I. Plasmid recombination

<table>
<thead>
<tr>
<th></th>
<th>Ampicillin</th>
<th>Ampicillin and Chloramphenicol</th>
<th>Recombination Frequency (%)</th>
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<tbody>
<tr>
<td>Signal joint (pJH200)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRAG1 wild type</td>
<td>1042</td>
<td>24</td>
<td>2.3</td>
</tr>
<tr>
<td>mRAG1 C325Y</td>
<td>1840</td>
<td>0</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Coding joint (pJH290)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRAG1 wild type</td>
<td>2461</td>
<td>37</td>
<td>1.5</td>
</tr>
<tr>
<td>mRAG1 C325Y</td>
<td>3275</td>
<td>0</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

FIGURE 1. mRAG1 C325Y supports reduced recombination. A. Top, SJ formation on recombination substrate pJH200 supported by pH548-derived mRAG1-expression vectors. Filled arrow, SJ-specific PCR product. B. Same as A except that pH548-derived mRAG1-expression vectors were used. C. SJ formation. D. CJ formation. E. DpnI-digested DNA from C and D was subjected to serial 2-fold dilution and amplified with primers that are not affected by recombination; open arrow, recombination-independent PCR product. W. T., Wild type.
that increased recombination supported by wild-type RAG1 \( \times 2 \)-to 4-fold (for example, compare A and B of Fig. 1). These findings are in agreement with previous studies indicating that RAG1 proteins with alterations to other conserved zinc-coordinating residues within the RING finger supported reduced SJ formation (19, 20). mRAG1 C325Y displayed no detectable SJ or CJ formation on recombination substrate pH299 (Fig. 1, C, SJ; D, CJ), which retains both SJ and CJ in the plasmid backbone. Thus, mRAG1 C325Y demonstrated at least a 50-fold reduction in the ability to support SJ formation and a defect in CJ formation of equal or greater severity.

**Defects in mRAG1<sub>218–389</sub> C325Y ubiquitin ligase activity and folding**

A fragment of mRAG1 spanning residues 218–389 acts as a ubiquitin ligase that promotes its own ubiquitylation in a reconstituted system including only purified components (26). mRAG1<sub>218–389</sub> C325Y was defective in this activity. The C325Y fragment showed reduced solubility when purified by our standard protocol (data not shown), so an alternate method was used for both the mutant and wild-type RAG1<sub>218–389</sub> fragments (see Materials and Methods). This had no effect on ubiquitin ligase activity of the wild-type RAG1 fragment in side by side assays (data not shown). Ubiquitin conjugation requires ubiquitin-activating and -conjugating enzymes in addition to the ubiquitin ligase (reviewed by Pickart; Ref. 28), and we demonstrated previously that RAG1 ubiquitin ligase activity was most robust in the presence of the ubiquitin-conjugating enzyme CDC34 (26). The wild-type RAG1 fragment promoted its own ubiquitylation even at the lowest levels of CDC34 tested (Fig. 2A, lanes 1–5), and at the highest levels of CDC34, greater than 20% of the wild type fragment was converted to a ubiquitylated form (Fig. 2A, lane 5). A very small but detectable fraction (~0.1%) of the C325Y fragment underwent ubiquitylation, but only at the highest concentration of CDC34 tested (Fig. 2A, lane 10).

The reduced ability of mRAG1<sub>218–389</sub> C325Y to functionally interact with CDC34 was most likely because of misfolding. Partial proteolysis confirmed that the C325Y substitution resulted in an alteration to the tertiary structure of the zinc-binding RING finger domain. Treatment with 200 ng of trypsin for 1–2 h resulted in nearly complete digestion of RAG1<sub>218–389</sub> (Fig. 2C, lanes 2 and 6). However, limited digestion with 20–40 ng trypsin produced a stable fragment of ~13.9 kDa (Fig. 2C, lanes 3 and 4 and 7 and 8), which was recovered and subjected to N-terminal sequencing. The first 12 amino acids (VSSKEVLK-KISN) corresponded to aa 254–265 of mRAG1, indicating that trypsin cleaved at mRAG1 R253. The size of the fragment indicated the C terminus to be either K377 or K380, which would produce fragments of 13.976 and 14.321 kDa, respectively. To ease further analysis by trypsinolysis, antisera was raised against the mRAG1 peptide VSSKEVLK-KISN. This Ab recognized both the mRAG1<sub>218–389</sub> fragment and the 13.9-kDa trypsinolysis product (Fig. 2D) and indicated that nearly all of the wild-type protein was quantitatively converted to the stable, 13.9-kDa product. A time course of trypsinolysis was performed to compare the relative sensitivities of mRAG1<sub>218–389</sub> wild type and C325Y. Over the course of 120 min, the 13.9-kDa fragment accumulated steadily in the reaction including wild-type protein (Fig. 2E, ◊). The C325Y protein, in contrast, showed a peak in production of the 13.9-kDa fragment between 30 and 60 min, after which time this species began to decrease slightly (Fig. 2E, △). In addition, whereas ~80% of the wild-type protein was proteolyzed after the 2-h incubation, only ~25% of the C325Y protein was susceptible (Fig. 2F). This suggests that the C325Y protein pool was a mixed population including a sizable proportion

![FIGURE 2](http://www.jimmunol.org/)  
Amino acid substitutions in the mRAG1 RING abrogate ubiquitin ligase activity. A. Ubiquitin ligase assays were performed as described in Materials and Methods using mRAG1<sub>218–389</sub> wild type or C325Y and CDC34 as indicated. Products were analyzed by Western blot with an Ab that recognizes the RAG1 construct (anti-Xpress-HRP, 1/1000). Filled arrows, ubiquitylated products; open arrow, unmodified mRAG1<sub>218–389</sub> protein pool was a mixed population including a sizable proportion...
that was resistant to proteolysis, perhaps in the form of marginally soluble aggregates, and a species that retained some native structure but was marginally less stable than the fully folded wild type and thus susceptible to further proteolysis. These data are consistent with previous circular dichroic measurements demonstrating that removal of zinc from this domain causes a loss of regular secondary structure (36). The reduced solubility of mRAG1 C325Y expression and cellular distribution

Normal mRAG1 C325Y expression and cellular distribution

In repeated trials, the steady state level of ectopic mRAG1 C325Y protein expression in pro-B cells was moderately increased relative to the wild type protein when measured 24 h after transfection (Fig. 3A). This difference was determined not to be significant based on paired t test analysis \( t_2 = -2.87; p = 0.10 \). At later time points, there was a substantial decrease in protein levels for both wild type and C325Y (data not shown). This suggested that the altered tertiary structure of the RING domain resulted in minimal destabilization of the full-length protein. We found that the wild-type protein supported recombination even when expressed from a less robust promoter (see Fig. 1A) and when the amount of transfected plasmid was reduced (see Fig. 5E and data not shown), confirming that moderate variations in mRAG1 C325Y expression could not account for the large decrease in recombination supported by this mutant.

Separation of cellular proteins into cytoplasmic, nuclear, and insoluble fractions revealed that a large proportion of the mRAG1 protein, both wild type and C325Y, was found in the insoluble fraction (Fig. 3B, lanes 4 and 8), in agreement with previously published results (37). Similar levels of mRAG1 wild-type and C325Y proteins could be found in the soluble nuclear fraction (Fig. 3B, lanes 3 and 7), but the proteins were also found in the low-salt cytoplasmic extraction. Distribution of lamin and copper/zinc superoxide dismutase proteins, markers for the nuclear matrix and cytoplasm, respectively, were used as controls (Fig. 3B). The appropriate distribution of these proteins indicated that the apparent presence of mRAG1 in the cytoplasm was not because of poor fractionation technique. Overall, the pattern of cellular distribution for the mRAG1 wild-type and C325Y proteins was very similar.

mRAG1 nuclear localization was confirmed by confocal microscopy. EGFP alone or mRAG1-EGFP wild-type or C325Y proteins were transiently expressed in NIH3T3 cells. The attachment of EGFP to the C terminus of mRAG1 had no impact on recombination (data not shown). When cells were transfected with pEGFP-N1, EGFP fluorescence was dispersed throughout the cell in both the nucleus and cytoplasm (Fig. 3C, left). When EGFP was fused in frame to the C terminus of either mRAG1 or mRAG1 C325Y, the distribution of EGFP fluorescence was entirely nuclear (Fig. 3C, middle and right). This indicated that both the mRAG1 wild-type and mRAG1 C325Y proteins were confined to the nucleus.

We saw a similar pattern in pro-B cells (data not shown). However, 4′,6′-diamidino-2-phenylindole staining indicated that the nucleus occupies a large percentage of the volume in these cells (data not shown); therefore, the pattern of nuclear localization of RAG1 vs whole-cell distribution of EGFP was more difficult to distinguish. FACs analysis of transiently transfected pro-B cells indicated that the levels of mRAG1-EGFP wild-type and C325Y expression were indistinguishable from one another but that both were expressed at a much lower level than EGFP alone (data not shown), again suggesting that the wild-type and mutant mRAG1 proteins are regulated in a similar manner. We cannot explain why mRAG1 was present in the cytoplasmic extract although the microscopy clearly indicated its nuclear localization, but this discordant pattern has been noted previously (37). Taken together, these data indicate that inappropriate compartmentalization could not account for the mRAG1 C325Y recombination defect.

Ubiquitin ligase activity and folding of mRAG1 P326G

To explore whether the inability of mRAG1 C325Y to support robust recombination was primarily due to the ubiquitin ligase deficiency or the folding defect, we examined the activity of an additional mutant. Silver et al. (20) found that substitutions in mRAG1 at either C328 (another zinc-coordinating residue) or P326 each reduced SJ formation by >10-fold when tested in the
The inability of mRAG1 P326G to support ubiquitin ligase activity despite the apparent structural integrity of the RING domain is likely due to a lack of interaction with the ubiquitin-conjugating enzyme. A proline at this position is highly conserved among both RAG1 and other RING domains (Fig. 4 B), both of which support ubiquitin ligase activity of the RAG1 RING domain (our unpublished observations). The Pavletich laboratory has shown that the equivalent proline in the cbl RING, P417, makes van der Waals contacts with P97 of the UbcH7 ubiquitin-conjugating enzyme. A proline at this position is highly conserved among both RAG1 and other RING domains (Fig. 4 A), where a bend is required to allow the cysteine side chains to interact optimally with zinc (24). A replacement at this position with glycine could theoretically still allow the requisite degree of bend in the polypeptide backbone. We found that partial proteolysis of mRAG1_218–389, P326G produced the 13.9-kDa fragment generated by digestion of the wild-type protein. The 13.9-kDa band accumulated throughout the time course of digestion in a manner similar to wild type (Fig. 2 E, □), although with slightly delayed kinetics, and nearly all of the full-length protein was converted to the stable 13.9-kDa species within the 2-h time course (Fig. 2 F). Thus, mRAG1_218–389 was able to maintain a native-like tertiary fold even with the P326G substitution.

Recombination defect in mRAG1 P326G

We found that the mRAG1 P326G substitution demonstrated a mild defect in recombination when expressed at relatively high levels, but the defect was more severe when RAG1 was limiting. As with mRAG1 C325Y, inappropriate cellular compartmentalization was ruled out as a cause for this defect, as mRAG1 P326G partitioned in a manner indistinguishable from wild type (Fig. 3 B, compare lanes 1–4 with lanes 9–12). Semiquantitative PCR of SJs generated on pH200 indicated a consistent 2- to 5-fold reduction in recombination relative to the wild-type protein (Fig. 5 A, compare lanes 3 and 6). Furthermore, mRAG1 P326G supported both SJ and CJ formation on pHJ299 (Fig. 5, B, SJ; C, CJ), although the reduction in CJ formation appeared to be more severe than that of SJ. Normal SJ formation on these substrates generates an ApaLI restriction site; therefore, ApaLI digestion can be used as an indication of accuracy of joining. SJ generated by either mRAG1 wild type or P326G could be digested to completion with ApaLI (Fig. 5 B), indicating that the P326G substitution had no effect on the accuracy of joining. Again, control amplifications performed on serially diluted DpmI-treated DNA indicated consistent levels of the replicated recombination substrate (Fig. 5, A, bottom, and D). To determine whether high levels of mRAG1 P326G protein may mask a more severe underlying recombination defect, we repeated the recombination assay using reduced quantities of the pJM071 wild-type and P326G vectors. mRAG1 wild type supported robust CJ formation even when the amount of vector was reduced by 20-fold from 10 to 0.5 μg (Fig. 5 E, lanes 2–5). The P326G variant showed a marked decrease in recombination activity under these conditions. CJ formation was barely detectable when the quantity of expression vector was reduced by only 10-fold (Fig. 5 E, lanes 6–9). A parallel experiment was performed under conditions used for detection of protein expression (Fig. 5 F); more plasmid was used in these experiments because of the greater number of cells transfected in each condition. This revealed that the levels of mRAG1 wild-type and P326G protein both decreased markedly as the amount of plasmid was decreased, whereas endogenous β-actin levels remained constant (Fig. 5 F, β). A 16-h exposure was required to detect the mRAG1 protein expressed after transfection with 1 μg of plasmid, whereas the protein expressed from 20 μg of plasmid could be easily detected after a 5-min exposure (Fig. 5 F, compare top and middle). Nevertheless, the expression of the two proteins was similar to one another at each level of plasmid tested. These data indicate that mRAG1 P326G was defective in CJ formation, but unlike mRAG1 C325Y, the recombination defect in mRAG1 P326G can be partly overcome when the protein was expressed at relatively high levels. We conclude from this that the recombination defect in mRAG1 C325Y can be partly explained by the ubiquitin ligase deficiency, but that misfolding of the mRAG1 C325Y RING domain also contributes strongly to the reduced ability of this protein to support recombination.

Discussion

RAG1 is a large protein comprising multiple biochemical activities, including specific and nonspecific DNA binding, DNA cleavage, RAG2 interaction, and ubiquitin ligase activity. The so-called core region of the protein has been subjected to fairly extensive study partly because of its ease of purification and partly because the core is competent for all of the known biochemical activities of RAG1 except for the ubiquitin ligase activity. Here we address the failure of a human RAG1 allele (7) with a missense mutation in the RING finger/ubiquitin ligase domain outside of the core to support B cell development. Our data confirm previous indications that an intact RING finger domain is required for normal levels of V(D)J recombination when recombination is being conducted by the full-length protein (18–20). We extend these observations by demonstrating that the RING finger substitution, mRAG1 C325Y, disrupts the tertiary fold of the RING domain, but that it is expressed and distributed in the cell similarly to the wild-type protein. Both
mRAG1 C325Y and an adjacent substitution that does not markedly disrupt folding, P326G, were profoundly defective in ubiquitin ligase activity in our reconstituted assay. However, mRAG1 C325Y demonstrated a more severe defect in recombination in pro-B cells, and unlike mRAG1 P326G, this defect could not be overcome by increasing the level of RAG1 expression. These data indicate that the severe recombination defect in mRAG1 C325Y is primarily due to misfolding of the RING domain. In addition, experiments with mRAG1 P326G suggest that ubiquitin ligase activity can contribute to recombination under conditions where RAG1 is limiting.

There are several possible explanations for the presence of T lymphocytes but not B lymphocytes in the patient expressing the hRAG1 C328Y variant. Maternal fetal engraftment, in which maternal T cells populate an immunocompromised infant, was definitively ruled out in this case (7). This confirms that the patient was able to independently generate T cells but not B cells. One possibility is that a correctly folded RING finger domain and/or ubiquitin ligase activity is specifically required for Ig but not TCR gene rearrangement. Alternatively, it may be required to activate the recombinase in pro-B cells. These seem unlikely because 1) mRAG1 C325Y was unable to support recombination of extrachromosomal substrates which are free of many of the constraints placed on the chromosomal recombination loci, 2) we and others have found that disruption of the RING finger results in reduced recombination in a variety of cell types (19, 20), and 3) the absolute numbers of both B and T cells in this patient were reduced relative to normal controls and other OS patients (7). Thus, overall recombination was reduced in both developing cell types, but the residual activity of the recombinase was sufficient to support limited rearrangement of the TCR loci. This suggests that a higher level of recombinase activity is required to support Ig gene rearrangement. In agreement with this, the level of recombinase activity present in recombining pro-/pre-B lymphocytes appears to be higher than that in pro-/pre-T lymphocytes (40, 41). Finally, we cannot rule out the possibility of secondary, compensatory mutations in the T cell population, as has been observed in at least one other OS case (17).

What is the function of RAG1’s N-TR? We and others have observed that substitutions that disrupt the RING finger domain within the N-TR can virtually eliminate recombination despite the entire region’s being dispensable for DNA cleavage (18–20). It has also been demonstrated that cysteine-rich and arginine/lysine-rich elements in the N-TR but outside the RING domain contribute to optimal levels of recombination activity (18, 42), and that the N-TR as a whole promotes SJ formation (43). Taken together, these data strongly suggest that the N-TR plays a regulatory role. One possible model is that a negative regulator maintains RAG1 in an inactive conformation, with the N-TR masking the active site within the RAG1 core or otherwise inhibiting RAG1 recombinase.
activity. This type of regulation could occur at any stage of recombination (e.g., DNA binding, pre- or postcleavage). The regulatory protein would be ubiquitylated in a RAG1-dependent manner under conditions conducive to recombination (Fig. 6A). If this is the case, the search for targets of RAG1 ubiquitin ligase activity is expected to lead to the identification of additional RAG1/2-regulatory proteins. Alternatively, autoubiquitylation of RAG1 could disrupt interaction with the regulator. If the negative regulator is limiting, overexpression of RAG1 would be expected to partially compensate for a ubiquitin ligase defect, allowing some of the protein to achieve an active conformation, as we see with mRAG1 P326G (Fig. 6B). Roman et al. (18) similarly observed that substitutions elsewhere in the N-TR had a more profound effect when RAG1 was limiting. The folding defect of mRAG1 C325Y would severely curtail its ability to achieve an active conformation under any circumstances (Fig. 6C). We also cannot rule out the possibility of a mild folding defect in mRAG1 P326G which may contribute to its recombination defect. In this model, deletion of the N-TR to create the RAG1 core is expected to have a relatively mild effect on recombination because the protein would be free of both the N-TR and negative regulators that bind it in trans (Fig. 6D).

Although this model is purely hypothetical at present, one intriguing candidate for the role of regulator is the nuclear import protein karyopherin subunit α1 (also called SRP-1, importin subunit α5, RAG1 cohort protein 2/RCH2). Karyopherin α1 interacts specifically with the basic region of RAG1 upstream of the RING domain (44), a region in which RAG1 has been shown to undergo autoubiquitylation in purified systems (26). This region is neither necessary nor sufficient for RAG1 nuclear localization (45), raising a question as to the role of interaction with karyopherin α1. We would propose that this interaction keeps RAG1 in an inactive state associated with the nuclear envelope. Release from karyopherin α1, possibly through autoubiquitylation of RAG1 in conjunction with karyopherin-releasing factors (46), would allow RAG1 to travel to the site of recombination. This model parallels the behavior of the IgH and Igκ loci, which are localized to the nuclear periphery in hematopoietic progenitors and pro-T cell, most likely through interaction with the nuclear lamin, and then move away from the periphery very early in pro-B cell development before the onset of recombination (47). Subnuclear compartmentalization may be a general regulatory theme in V(D)J recombination. In addition, there are now several examples of karyopherins acting as regulators of nuclear function outside of their roles in nuclear transport (48, 49).

Potential autoregulation of RAG1 by elements within the N-TR mirrors the autoregulation that exists in the core itself. The Rodgers laboratory has demonstrated that the RAG1 C-terminal domain (aa 761–979) can suppress ssDNA cleavage activity resident in the RAG1 central domain (aa 528–760) in cis or trans, whereas RAG2 enhances ssDNA cleavage and activates dsDNA cleavage (50). These and other data present a picture of RAG1 as an inherently active ssDNA endonuclease. This potentially damaging activity is subjected to multilayered biochemical and spatiotemporal regulation by elements within RAG1 as well as RAG2 and possibly other proteins. The RAG1 ubiquitin ligase activity may add an additional layer of regulation, or it may be used during another stage of development. Examination of recombination on the chromosomal loci as well as the ability of mutant alleles to support lymphocyte development in animal models will be necessary to ascertain how elements within the N-TR fit into this complicated regulatory milieu.

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Disclosures
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References


