REVIEW

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The roles of the RAG1 and RAG2 "non-core" regions in V(D)J recombination and lymphocyte development

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Abstract

The enormous repertoire of the vertebrate specific immune system relies on the rearrangement of discrete gene segments into intact antigen receptor genes during the early stages of B- and T-cell development. This V(D)J recombination is initiated by a lymphoid-specific recombinase comprising the RAG1 and RAG2 proteins, which introduces double-strand breaks in the DNA adjacent to the coding segments. Much of the biochemical research into V(D)J recombination has focused on truncated or "core" fragments of RAG1 and RAG2, which lack approximately one third of the amino acids from each. However, genetic analyses of SCID and Omenn syndrome patients indicate that residues outside the cores are essential to normal immune development. This is in agreement with the striking degree of conservation across all vertebrate classes in certain non-core domains. Work from multiple laboratories has shed light on activities resident within these domains, including ubiquitin ligase activity and KPNA1 binding by the RING finger domain of RAG1 and the recognition of specific chromatin modifications as well as phosphoinositide binding by the PHD module of RAG2. In addition, elements outside of the cores are necessary for regulated protein expression and turnover. Here the current state of knowledge is reviewed regarding the non-core regions of RAG1 and RAG2 and how these findings contribute to our broader understanding of recombination.

Key words: V(D)J recombination, RAG1, RAG2, RING finger, PHD domain/plant homeodomain.

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INTRODUCTION

Definition of "core" regions of proteins is a common and useful research technique. A protein is progressively shortened until the minimal region necessary and sufficient to perform in some defined assay is delineated. The elimination of "extraneous" or "non-essential" portions of the protein has the advantage that the minimum activities required for biological function (under laboratory conditions) can be identified. But even the most exhaustive analysis of the core activities cannot provide a complete picture of the intact protein in its normal milieu. This review is dedicated to the non-core (*nc*) regions of the RAG1 and RAG2 proteins (Sadofsky et al. 1994; Sadofsky et al. 1993; Silver et al. 1993), which in their intact form comprise the V(D)J recombinase (Gellert 2002). While extensive and ongoing biochemical analysis of the core (*c*) RAG1/2, catalogued in a number of excellent reviews (Gellert 2002; Schatz and Spanopoulou 2005), has provided fascinating insights into the biochemical mechanisms and regulation of DNA binding, cleavage, and joining, a growing body of evidence indicates that activities essential to normal immune development lie outside the core regions. Consistent with this, the *nc* regions have remained intact since the Cambrian period, during approximately 500 million years of jawed vertebrate diversification.

V(D)J RECOMBINATION

Combinatorial immunity is the hallmark of the vertebrate-specific immune system (Tonegawa 1983). In the germ line, immunoglobulin (Ig) and T cell receptor (TCR) genes are divided into short discrete modules including multiple families of variable (V) and fewer joining (J) segments. V and J are further separated by

diversity (D) segments on the Ig heavy chain as well as TCR δ and ß loci. Each V, D, and J coding segment is flanked by a recombination signal sequence (RSS) composed of conserved heptamer and nonamer motifs separated by non-conserved spacers of either 12 or 23 bases. The coding segments are brought together to form intact antigen receptor genes by the process of V(D)J recombination, and only coding segments flanked by RSS with non-identical spacer lengths can be combined (Tonegawa 1983). This "12/23 rule" helps to prevent non-productive rearrangement, such as V to V or J to J. Recombination occurs in a strict temporal order, with D to J recombination occurring prior to V to DJ on the heavy chain and heavy chain recombination preceding that of light chains (Yancopoulos and Alt 1986). Moreover, TCR rearrangement is restricted to the early stages of T-cell development and Ig rearrangement is restricted to early B cells. Adherence to this regimen relies on both restricted expression of the recombinase and cell-specific accessibility of the loci. Recombination of extra-chromosomal substrates including RSS can occur in any cell type if RAG1 and RAG2 are provided ectopically, but ectopic expression of the recombinase alone will not result in rearrangement at the antigen receptor loci except in the appropriate immune lineages.

The RAG1/2 recombinase is the only lymphoid-specific protein complex required for V(D)J recombination (Oettinger et al. 1990; Schatz et al. 1989). The intact murine proteins are 1040 and 527 amino acids long, respectively, with those from other species varying in length by only 3-10 amino acids. As much of the biochemical work has been performed using the murine proteins, murine numbering will be used throughout this review unless otherwise noted. The core of RAG1 (*c*RAG1) spans amino acids 384-1008 (Sadofsky et al. 1993; Silver et al. 1993) and the RAG2 core (*c*RAG2) spans amino acids 1-387 (Sadofsky et al. 1994; Silver et al. 1993). There is currently no confirmed structural data available for either core region, but domains outside the cores of both proteins have been crystallized and/or subjected to NMR analysis. This includes the zinc-binding C_3HC_4 RING finger motif and the associated C_2H_2 zinc finger spanning amino acids 264-389 of *nc*RAG1 and the zinc-binding plant homeodomain (PHD) module spanning amino acids 414-487 of *nc*RAG2 (Bellon et al. 1997; Elkin et al. 2005; Matthews et al. 2007; Ramon-Maiques et al. 2007). Other RAG2 *nc* regions of significance are the "hinge" region between the core and PHD and the extreme carboxy--terminal end (amino acids 488-527). The RAG1 *nc* includes a basic region upstream of the RING, several additional cysteine-rich motifs, and a highly conserved segment in the far N-terminus.

RAG1/2 initiates recombination by introducing double-stranded breaks in the DNA between the RSS and coding segment (Fig. 1A). In the first step of cleavage, a nick is introduced, leaving a free 3-prime hydroxyl on the coding DNA (van Gent et al. 1996a). In the second step, this 3'-OH attacks the phosphodiester bond on the opposite strand in a direct transesterification reaction (van Gent et al. 1996a), leaving a covalently closed hairpin on the end of the coding segment and a blunt-cut RSS end. In the presence of Mg^{2+}, the likely physiological cofactor, the transesterification step of cleavage can only occur efficiently in the context of a 12/23 synaptic complex (Eastman et al. 1996; van Gent et al. 1996b). Thus RAG1/2 establishes the 12/23 rule during cleavage. While much of the biochemistry of cleavage was originally derived using *c*RAG1/2, it has since been recapitulated using purified full-length proteins (Elkin et al. 2003; Swanson et al. 2004; Tsai and Schatz 2003). In addition, hairpin-ended coding segments and blunt-cut RSS ends have been isolated from cells undergoing V(D)J recombination (Roth et al. 1992; Roth et al. 1993). After cleavage in the cell, the coding ends are rapidly opened and joined together in a process dependent on the general non-homologous end-joining (NHEJ) DNA repair machinery (Ramsden and Gellert 1995; Taccioli et al. 1993). Signal ends remain tightly bound to the recombinase in a post-cleavage complex (SEC) which must be remodeled before the ends can be joined (Agrawal and Schatz 1997; Jiang et al. 2004; Jones and Gellert 2001). The SEC has been shown to participate in additional DNA transactions (Fig. 1B and C), including transposition (Agrawal et al. 1998; Hiom et al. 1998), i.e. the attack of non-specific DNA, and open-and-shut/hybrid joint formation (Lewis et al. 1988), i.e. the re-attack of the hairpin coding ends. Only the latter has been shown to occur with any frequency in the cell.

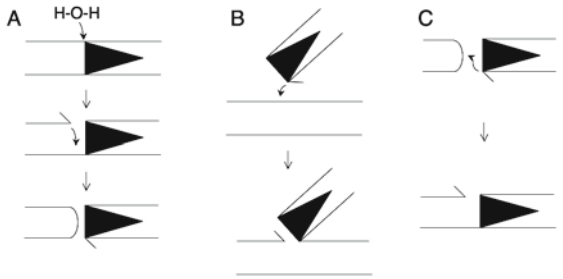

Fig. 1. RAG1/2-catalyzed reactions. Triangles represent RSS. **A** – DNA cleavage. Cleavage is initiated by hydrolytic nicking. The free 3'-OH then attacks the opposite strand to create a double-strand break in the DNA. Cleavage normally takes place in a synaptic complex including two RSS (not shown). **B** – transposition. The blunt RSS end attacks nonspecific DNA. This occurs in the context of a synaptic complex including two RSS, and both may become covalently attacked to target DNA (not shown). **C** – hybrid and open-and-shut joint formation. The RSS end attacks the hairpin-ended coding DNA from which it was cleaved (open-and-shut) or from which its partner RSS was cleaved (hybrid).

Obviously, many of the biochemical activities essential for recombination are present in the core regions, including RSS-specific DNA binding and cleavage. However, they do not fully recapitulate the activity of the intact recombinase (Tables 1–3; check the following urls for an updated list of RAG1 and RAG2 mutations identified in

Table 1. Expression and recombination activity of selected RAG1 variants relative to the intact wild type (W.T.)

RAG1	Expression	Recombination
cRAG1	up	~10–40% on extra-chromosomal substrates (Sadofsky et al. 1993; Silver et al. 1993) ~10% on IgH locus (Roman et al. 1997) mature B and T cells present in mouse, reduced overall recombination, increased aberrant joints (Dudley et al. 2003; Talukder et al. 2004)
RAG1Δ13-79	up	>cRAG1 (Roman et al. 1997)
RAG1Δ13-135		<cRAG1 (Roman et al. 1997)
RAG1Δ299-330		<1% (Roman et al. 1997)
C110A		<2% (Roman et al. 1997)
C113A		<2% (Roman et al. 1997)
C290S	~W.T.	~5% (Silver et al. 1993)
C293S/H307L/C313S	down	<2% (Sadofsky et al. 1993)
C325Y	~W.T.	<1% (Simkus et al. 2007)
P326G	~W.T.	~10%, <1% under limiting conditions (Silver et al. 1993; Simkus et al. 2007)
C328S	~W.T.	~6% (Silver et al. 1993)
hC328Y		OS (Villa et al. 2001)
hK136Q		OS (Sobacchi et al. 2006)
hC192Y		OS (Sobacchi et al. 2006)
hR314W		B/T reduced immunodeficiency with granulomas (Schuetz et al. 2008)

All substitutions refer to the murine protein unless designated human (h).

Table 2. Expression and recombination activity of selected RAG2 variants relative to the intact wild type (W.T.)

RAG2	Expression	Recombination
cRAG2	up	~40–100% on extra-chromosomal substrates (Silver et al. 1993; Sadofsky et al. 1994) block in IgH V to DJ rearrangement in mouse (Liang et al. 2002; Akamatsu et al. 2003)
RAG2Δ388-413		~100% (Silver et al. 1993)
RAG2Δ521-527		~250% (Elkin et al. 2005)
Y402A		reduced IgH V to DJ (West et al. 2005)
N403A		reduced IgH V to DJ (West et al. 2005)
D406A		reduced IgH V to DJ (West et al. 2005)
E407A		reduced IgH V to DJ (West et al. 2005)
Y415A		reduced IgH D to J (Matthews et al. 2007)
M443A		reduced IgH D to J (Matthews et al. 2007)
W453A		reduced IgH D to J (Matthews et al. 2007)
T490A	up	supports recombination no G1/S-specific destruction of RAG2 accumulation of aberrant products in mouse thymocytes (Lee and Desiderio1999) persistence of SEC throughout cell cycle (Jiang et al. 2004)
hW416L		OS (Sobacchi et al. 2006)
hK440L		OS (Sobacchi et al. 2006)
hW453R		OS (Noordzij et al. 2002)
hN474S		SCID with maternal fetal engraftment (Villa et al. 2001)
hC478Y		SCID (Villa et al. 2001)
hH481P		SCID (Noordzij et al. 2002)
M502V		OS (Sobacchi et al. 2006)

All substitutions refer to the murine protein unless designated human (h).

Table 3. Effects of various combinations of RAG proteins on the relative production of signal ends (SE), coding ends (CE), signal joints (SJ), and coding joints (CJ) (Steen et al. 1999)

RAGs	SE	CE	SJ	CJ
*fl*RAG1/*fl*RAG2	1.0	1.0	1.0	1.0
*c*RAG1/*c*RAG2	9.8±3.7	1.0	0.3–0.5	0.1–0.2
*c*RAG1/*fl*RAG2	3.0±1.2	1.0		
*fl*RAG1/*c*RAG2	2.2±0.5	1.0		

human patients: www.uta.fi/imt/bioinfo/RAG1base and www.uta.fi/imt/bioinfo/RAG2base) (Piirila et al. 2006). *c*RAG1/2 can support recombination of extra-chromosomal RSS-containing substrates at about 10–40% of the level of the intact recombinase (Sadofsky et al. 1994; Sadofsky et al. 1993; Silver et al. 1993), despite being expressed at much higher levels. In addition, *c*RAG2 has a stage-specific defect in chromosomal recombination, being competent for D to J but not V to DJ joining (Akamatsu et al. 2003), which leads to a deficit in mature lymphocytes in the *c*RAG2 knock-in mouse and an increase in the number of aberrant joints (Akamatsu et al. 2003; Liang et al. 2002; Talukder et al. 2004). *c*RAG1 is also partially defective in chromosomal recombination (Roman et al. 1997), and the knock-in mouse displays a phenotype consistent with an overall reduced level of rearrangement and an increase in aberrant joints (Dudley et al. 2003; Talukder et al. 2004). In an otherwise intact protein, mutations in the *nc* regions can lead to reductions in recombination far more severe than that seen with deletion of the entire *nc* regions (Roman et al. 1997; Sadofsky et al. 1994; Sadofsky et al. 1993; Silver et al. 1993; Simkus et al. 2007). Furthermore, defects associated with mutations in *nc*RAG1 become more pronounced when the protein is present at limiting levels (Roman et al. 1997; Simkus et al. 2007), a situation likely to mimic normal recombination conditions (Leu and Schatz 1995). Many human patients with primary immune deficiencies have been identified with amino-acid substitutions outside the core. These include patients with B/T-negative severe combined immune deficiency (SCID; human RAG2 C478Y and H481P), Omenn syndrome (OS), a B-negative/T-oligoclonal variant of SCID (human RAG1 K136Q, C192Y, C328Y, human RAG2 W416L, K440N, W453R, and M502V) (Noordzij et al. 2002; Noordzij et al. 2000; Sobacchi et al. 2006; Villa et al. 2001), and a syndrome associated with limited maturation of B and T cells coupled with granulomas (human RAG1 R314W) (Schuetz et al. 2008). Possible biochemical explanations for some of these disease phenotypes will be provided later in the review.

EVOLUTION AND CONSERVATION OF THE RAG PROTEINS

The biochemical activities of the RAG1/2 recombinase are similar to those exhibited by transposase proteins (Agrawal et al. 1998; Hiom et al. 1998), and the RSS are reminiscent of the terminal repeats that flank transposable elements (Sakano et al. 1979). For decades it has been a working hypothesis in the field that combinatorial immunity evolved following horizontal transfer of an ancestral RAG1/2 transposase into a primordial antigen receptor gene, followed by decoupling of the transposase from its ends and reiterative expansion of the now segmented receptor (Thompson 1995). There is now strong homology-based evidence that the core region of RAG1 is a member of the *transib* family of transposons (Kapitonov and Jurka 2005), which is widely distributed in nematodes, insects, echinoderms, cnidiarians, and fungi, although apparently not in chordates or plants (Kapitonov and Jurka 2003). Furthermore, degenerate but identifiable *c*RAG1-like proteins divorced for the RSS-like *transib* ends have been identified in hydra and sea anemone (cnidiarians) and the deuterostomes sea urchin (echinoderm) and amphioxus (cephalochordate), although not in agnathans, the closest living relatives of the jawed vertebrates (Fugmann et al. 2006; Kapitonov and Jurka 2005).

The *transib* transposases and *c*RAG1-like proteins so far identified appear to lack the *nc*RAG1 region. However, all but the first 100 amino acids of *nc*RAG1 bears an intriguing homology to sea urchin and amphioxus proteins of unknown function, suggesting the intact RAG1 recombinase may have been assembled from distinct genes in an early deuterostome (Kapitonov and Jurka 2005). This is further supported by the observation that teleosts retain an intron between these two regions (Willett et al. 1997). Furthermore, the two parts of the protein, core and non-core, have been under different levels of purifying selection during the diversification of jawed vertebrates. The core has been conserved to an extreme degree (73% identity, 91% similarity; Fig. 2), while only certain key residues in *nc*RAG1, such as those that coordinate zinc, have been subject to the same degree of purifying pressure and the remainder has been allowed to diversify (21% identity, 54% similarity). The presence of this region in all modern RAG1 proteins suggests that the addition of the *nc*RAG1 modules(s) was a vital part of co-opting the ancestral transposase into performing its function in combinatorial immunity.

To further complicate the evolutionary picture, no *transib* equivalent of RAG2 has been identified. This suggests that either 1) the direct *transib* ancestor of RAG1/2 included a RAG2-like accessory protein, but no remnants of this family member remain in modern genomes, or 2) RAG2, like *nc*RAG1, is derived from a cellular ancestor. A homologue of RAG2 with some similar properties has been identified in sea urchin (Fugmann et al. 2006; Wilson et al. 2008). Unlike RAG1, there is no obvious distinction between the core and *nc* portions of RAG2 based on conservation, with the entire length of the protein showing roughly 50% identity across vertebrate classes (Fig. 2).

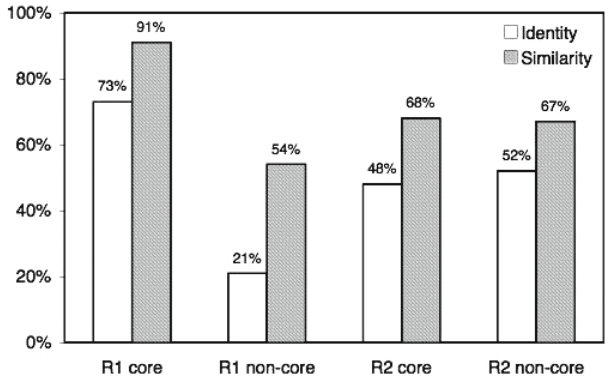

Fig. 2. Conservation of RAG1/2 core and non-core regions. Representative RAG1 (R1) and RAG2 (R2) proteins from mouse (NP_033045.2, CAM20887), chicken (NP_001026359.1, XP_421091), Xenopus (Q91829.1, AAI29720), and zebrafish (NP_571464.1, NP_571460) were aligned using Clustal W and the degrees of similarity and identity were calculated.

BIOCHEMISTRY OF THE RAG1 N-TERMINAL REGION

*nc*RAG1 spans amino acids 1-383 on the N-terminus and 1009-1040 in the C-terminus, but we will focus on the N-terminal portions as relatively little is known about the far C-terminus. Mutations within *nc*RAG1 are known to abrogate recombination of extra-chromosomal substrates (Sadofsky et al. 1993; Silver et al. 1993), and RAG1 alleles detected in OS and other immune-deficient patients include both N-terminally truncated proteins and *nc*RAG1 missense mutations (Noordzij et al. 2000; Santagata et al. 2000; Schuetz et al. 2008; Sobacchi et al. 2006; Villa et al. 2001), demonstrating that *nc*RAG1 is crucial to normal lymphocyte development. Regions of the N-terminus important for RAG1 function include a series of lysine/arginine-rich basic motifs spanning amino acids 218-264 (McMahan et al. 1997), which are bound by the karyopherin alpha 1 (KPNA1) protein (Cortes et al. 1994), and a zinc-binding RING finger domain (Rodgers et al. 1996) with ubiquitin ligase activity (Jones and Gellert 2003; Yurchenko et al. 2003).

RAG1 RING finger domain and other zinc-binding regions

Sequence analysis of the RAG1 protein indicates the presence of multiple zinc-binding motifs (Rodgers et al. 1996; Schatz et al. 1989). A fragment spanning amino acids 87-217 was recently found to bind two zinc ions (Rodgers, personal communication), and substitutions at conserved cysteine residues within this region (C110A, C113A) disrupt recombination on extra-chromosomal substrates (Roman et al. 1997). RAG1 has a C_3HC_4 RING finger (amino acids 287-351) which together with an adjacent zinc finger form a single integrated domain (Rodgers et al. 1996). The structure of the RAG1 RING is unusual in that it coordinates three as opposed to two Zn ions (Bellon et al. 1997). The first and second Zn ions are coordinated by canonical binding residues shared among all RING domains, and the third ion is coordinated by three noncanonical residues upstream (H270, C266, H295) and one canonical zinc-binding residue (C293) (Fig. 3A). More commonly, RING finger proteins are stabilized by coordination of two Zn ions in a cross-brace motif, as depicted for cbl (Fig. 3B) (Zheng et al. 2000). The presence of the unique bi-nuclear zinc cluster does not appreciably disrupt the overall cross-brace architecture of the RAG1 RING domain, and it can be aligned with the cbl RING with a root mean square deviation of 1.13 Å across 32 C-alpha pairs (authors' unpublished observations).

Ubiquitylation activity

Many RING-containing proteins exhibit ubiquitin ligase activity (Jackson et al. 2000; Weissman 2001). Ubiquitylation, the covalent attachment of the highly conserved 76-amino-acid ubiquitin protein to target proteins, plays regulatory roles in virtually every biochemical system in eukaryotes (Pickart 2001). Conjugation involves a multi-step cascade including a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), which is primarily responsible for target specificity. Mono-ubiquitylation at one or several sites can alter protein function and/or localization

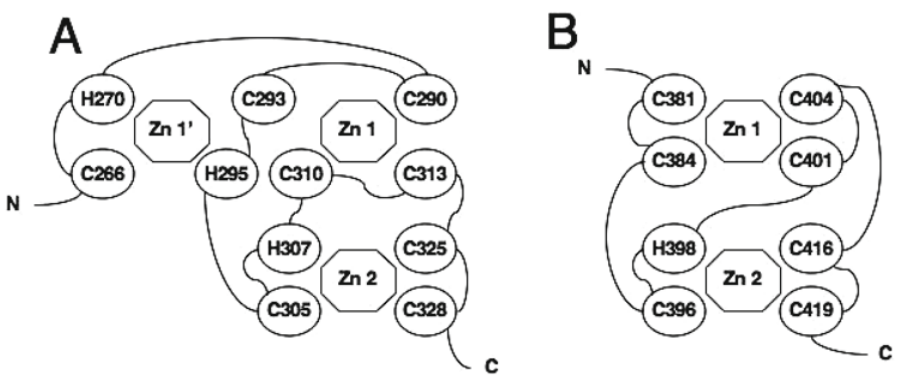

Fig. 3. Diagram of the RAG1 and cbl zinc-binding domains. **A** – zinc-binding residues of the RAG1 RING domain including the coordination of the third zinc ion by the non-canonical binding residues C266, H270, H295, and C293 (canonical). The first zinc site is coordinated by residues C290, C293, C310, and C313. The second zinc site is coordinated by residues C305, H307, C325, and C328. **B** – the cbl diagram represents a classic RING domain with the first zinc coordinated by C381, C384, C401, and C404. The second zinc is coordinated by residues C395, H398, C416, and C419.

(Pickart 2000). Ubiquitin can also be self-conjugated, creating poly-ubiquitin chains that usually target the modified protein for degradation by the 26S proteasome.

Isolated fragments of RAG1 including the RING finger region exhibit ubiquitin ligase activity and interact with multiple E2 enzymes to promote ubiquitylation of model substrates *in trans* (Yurchenko et al. 2003) and auto-ubiquitylation at several conserved lysine residues in the upstream basic region (Jones and Gellert 2003). The effects of RAG2 on this activity and the ability of purified full-length RAG1 to act as a ubiquitin ligase have not been determined. Ectopically expressed full--length RAG1 does undergo intracellular ubiquitylation regardless of the presence of RAG2 (Jones and Gellert 2003), but it is not known whether this occurs with endogenously expressed protein or whether RAG1 E3 activity is required. Amino-acid substitutions in conserved zinc-coordination sites disrupt recombination in model cellular systems (Roman et al. 1997; Sadofsky et al. 1993; Silver et al. 1993; Simkus et al. 2007) and two missense mutations in *nc*RAG1 associated with human immunodeficiency (human RAG1 C328Y and R314W) occur in the RING finger domain. We recently demonstrated that an equivalent substitution in murine RAG1 (C325Y) resulted in the loss of structural integrity and E3 activity in a reconstituted system and interfered with the ability of full-length RAG1 to support intracellular recombination (Simkus et al. 2007). To determine whether E3 activity was absolutely required for robust recombination, we tested an additional E3-incompetent substitution mutant (P326G) with apparently normal tertiary structure. RAG1[P326G] could support recombination of extra-chromosomal substrates, but only when present at very high levels. We have since found a correlation between E3 activity and intracellular recombination that is apparent only when RAG1 is limiting.

One possible model to explain these results is that *nc*RAG1 is bound by a negative regulator that must undergo RAG1-dependent ubiquitylation and destruction to activate recombination (Fig. 4). Over-expression of an E3-incompetent allele such as P326G would overwhelm limiting levels of the regulator, allowing recombination to occur, but this allele would display a profound defect in recombination when expressed at lower levels. Hypothetically, the folding defect in C325Y prevents it from being able to attain an active conformation at any level, regardless of the presence of the regulator. *c*RAG1 would be independent of the regulator altogether, being able neither to bind nor ubiquitylate it.

KPNA1 binding and ubiquitylation

RAG1 has been shown to bind to several cellular proteins in addition to RAG2, including KPNA1 (a.k.a. importin alpha 5, SRP1, and RAG cohort protein 2 (Cortes et al. 1994)), karyopherin alpha 2 (KPNA2, a.k.a. RAG cohort protein 1 (Cuomo et al. 1994)), and possibly the NHEJ factor Ku (Raval et al. 2008). KPNA2 binds to a nuclear localization signal within the core and is necessary and sufficient for nuclear import of RAG1 (Spanopoulou et al. 1995). KPNA1 binds to the basic region upstream of the *nc*RAG1 RING. Although mutations in this motif have been demonstrated to alter recombination activity (McMahan et al. 1997), interaction with KPNA1 is not required for import (Spanopoulou et al. 1995). Binding to KPNA1 does, however, influence sub--nuclear localization of RAG1 (Spanopoulou et al. 1995). RAG1 bound to KPNA1 displays a punctate or speckled pattern within the nucleus, while mutants that do not bind KPNA1 are distributed diffusely throughout the nucleus (Spanopoulou et al. 1995). These speckles are too numerous to represent active recombination complexes, which are generally confined to only two per cell (Chen et al. 2000). V(D)J recombination is already known to be regulated through subnuclear localization of the recombination loci (Kosak et al. 2002). Sequestration via interaction with KPNA1 could extend this theme to the recombinase itself.

We recently demonstrated that a fragment of *nc*RAG1 spanning the ubiquitin ligase domain and upstream basic region (amino acids 218-389) promoted ubiquitylation of purified KPNA1 (Simkus et al. 2008). This reaction was best supported by the E2 enzymes UbcH2/Rad6 and UbcH5a and required E1, ubiquitin, and ATP. Ubiquitylation of KPNA1 required the basic region spanning RAG1 amino acids 218-263 to which

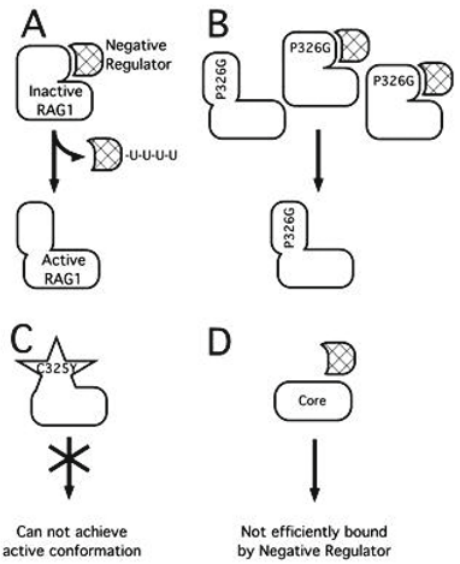

Fig. 4. Model for the regulation of recombination by RAG1 E3 activity. Negative regulator maintains RAG1 in an inactive conformation with the *nc* masking the active site within the RAG1 core or otherwise inhibiting RAG1 recombinase activity. **A** – the regulatory protein would be ubiquitylated (U) in a RAG1-dependent manner under conditions conducive to recombination. **B** – if the negative regulator is limiting, over-expression of RAG1 would be expected to partially compensate for a ubiquitin ligase defect, allowing some of the protein to achieve an active conformation, as seen with RAG1[P326G]. **C** – the folding defect of RAG1[C325Y] would severely curtail its ability to achieve an active conformation under any circumstances. **D** – RAG1 core has a relatively mild defect in recombination because the protein would not be bound by the negative regulator. Used with permission (Simkus et al. 2007).

KPNA1 binds, but RAG1 was still able to undergo auto-ubiquitylation in this region even in the presence of KPNA1. Future experiments will probe whether KPNA1 is the negative regulator predicted by our model.

Nuclear import

Two mutations associated with Omenn syndrome, Δ368A/369A and Δ887A in human RAG1, result in frame-shifted alleles that produce premature truncation after amino acids P85 and S258, respectively. It has been shown in both cases that initiation at internal methionines downstream of these mutations (M183 and M263, respectively) produces N-terminally truncated RAG1 proteins (Santagata et al. 2000). When expressed as recombinant proteins, RAG1 [183-1040] and [263-1040] support recombination of extra-chromosomal substrates at 100 and 10%, respectively, of the level of the wild type. However, the mutant OS alleles demonstrate a more severe defect in recombination. This appears to be the result of mis-localization of the proteins produced from the Δ368A/369A and Δ887A alleles. *nc*RAG1 has been shown to be dispensable for nuclear localization of RAG1 (Spanopoulou et al. 1995), even in the absence of RAG2. It is therefore possible that the presence of the C-terminally truncated proteins (RAG1 1-85 and 1-258), each of which expresses additional aberrant residues, may somehow interfere with proper localization of the N-terminally truncated RAG1 proteins, which are expressed at abnormally low levels to begin with. A combination of mis-localization, reduced expression, and reduced activity are likely to explain the defect in these OS patients.

BIOCHEMISTRY OF THE RAG2 C-TERMINUS

Like *nc*RAG1, multiple biochemical functions reside in *nc*RAG2 (amino acids 388-527), including chromatin and phosphoinositide binding and regulation of protein turnover (Elkin et al. 2005; Lin and Desiderio 1994; Liu et al. 2007; Matthews et al. 2007; Ramon-Maiques et al. 2007; West et al. 2005). Mice with a knock-in of *c*RAG2 fail to carry out V to DJ rearrangement (Akamatsu et al. 2003), and evidence from human patients indicates that the PHD module in *nc*RAG2 is required for normal immune development (Sobacchi et al. 2006; Villa et al. 2001).

Chromatin binding

One essential function of *nc*RAG2 is chromatin binding. While *c*RAG1/2 is competent for recombination on naked substrates, sequestration of RSS within nucleosomes prevents access by the recombinase (Kwon et al. 1998; Kwon et al. 2000). Initial studies indicated that the presence of *nc*RAG2 had no specific role in accessing SWI/SNF-remodeled V(D)J templates in a reconstituted cell-free system (Patenge et al. 2004). However, the presence of an intact *nc*RAG2 PHD domain has since been shown to be required for robust intracellular recombination of chromatinized substrates (Matthews et al. 2007). The apparent conflict with the earlier study can be explained by the observation that the *nc*RAG2 PHD module recognizes histone modifications distinct from those induced by SWI/SNF remodeling.

The *nc*RAG2 PHD module has been subjected to extensive structural analysis both free and bound to an N-terminal peptide of histone H3 trimethylated on lysine 4 (H3-K4me3; Fig. 5) as well as to other modified H3 peptides (Matthews et al. 2007; Ramon-Maiques et al. 2007). The module adopts a non-canonical PHD fold including two zinc ions coordinated by a conserved motif including five cysteine and three histidine residues in an interleaved configuration (Elkin et al. 2005; Matthews et al. 2007; Ramon-Maiques et al. 2007). The *nc*RAG2 PHD lacks the "aromatic cage" normally associated with trimethylated lysine residue-binding sites, instead including an aromatic cleft or channel which is open at the top and side (Matthews et al. 2007; Ramon-Maiques et al. 2007). In the uncomplexed structure, this cleft is occupied by a *cis*-peptide formed from eight residues carried over from the expression vector, with a proline residue mimicking many of the contacts otherwise provided by the tri-methyl lysine (Ramon-Maiques et al. 2007). One hypothesis is that the PHD may bind to a flexible proline-containing segment of *c*RAG2 in an auto-inhibitory capacity, possibly RAG2 P230, another residue found to be substituted in OS patients. In this model, binding to modified histones would both relieve this inhibition and recruit the recombinase to the site(s) of recombination.

The isolated *nc*RAG2 PHD module (amino acids 414-487) binds with relatively high affinity (K_d ~4 μM) to H3-K4me3, but with lower affinity to the di-, mono-, and non-methylated forms of the protein (K_d ~60, 120,

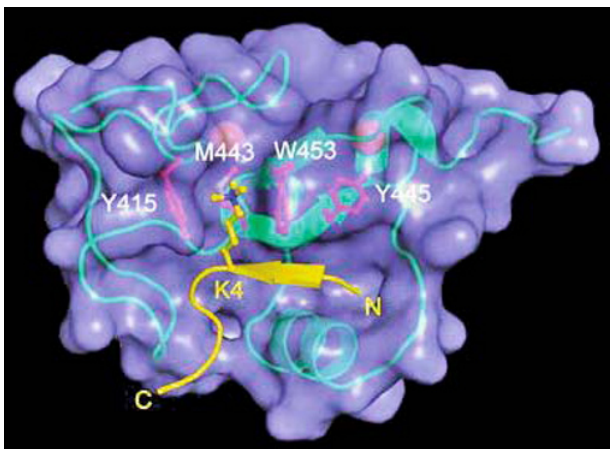

Fig. 5. Crystal structure of *nc*RAG2 PHD bound to H3-K4me3. H3 peptide is highlighted in yellow. The RAG2 PHD backbone is shown as a green ribbon encased in a purple molecular surface. Amino-acid side-chains essential for binding are highlighted. Y415, M433, and W453 form the aromatic channel. Y445 also makes favorable contacts with H4-R2me2 (not shown). Used with permission (Ramon-Maiques et al. 2007).

and 500 μM, respectively) (Liu et al. 2007; Matthews et al. 2007; Ramon-Maiques et al. 2007). The domain also binds to native HeLa cell nucleosomes but not to reconstituted recombinant nucleosomes (Matthews et al. 2007), indicating that modifications occurring in the cell are necessary for binding. Affinity is further enhanced by symmetric dimethylation of H3 arginine 2 (H3--R2me2/K4me3), thus providing the first example of a single domain that binds preferentially to a doubly modified histone (Ramon-Maiques et al. 2007). While other PHD-containing proteins such as ING2 bind to H3-K3me3 with higher affinity than does the *nc*RAG2 PHD, this binding is usually blocked by methylation of H3-R2. A single amino acid in the *nc*RAG2 PHD explains the preferential binding to H3-R2me2. A tyrosine at position 445 replaces a carboxylate group at this position in other PHDs. The carboxylate forms a salt bridge with unmodified H3-R2; thus methylation at this position decreases affinity. T445, on the other hand, forms favorable interactions with H3-R2me2, and no salt bridge is formed with unmodified H3-R2. It is proposed that preferential binding to the additional modification at H3-R2 may enhance both affinity and specificity, allowing RAG2 to compete effectively for binding at the recombination loci.

All of the amino-acid substitutions in *nc*RAG2 associated with immunodeficiency in human patients occur in the PHD module (Noordzij et al. 2002; Sobacchi et al. 2006; Villa et al. 2001). Two that disrupt zinc coordination (C478Y, H481P), and thus structural integrity of the domain, lead to SCID. A substitution associated with OS (W453R) abrogates binding to modified H3 without disrupting the structural integrity of the domain. Two additional OS substitutions are predicted to disrupt the hydrogen-bonding network with modified H3 (K440N) and the stability of the aromatic channel that binds trimethylated lysine (W416L) (Matthews et al. 2007; Ramon-Maiques et al. 2007).

Methylation of H3-K4 is indeed required for robust recombination on chromatinized templates. On templates with nucleosomes positioned to obscure the RSS, recombination can be reduced by either knocking down the H3-K4 methytransferase WDR5 or over-expression of the demethylase SMCX (Matthews et al. 2007). Chromatin immunoprecipitation assays indicate that binding of intact RAG2 to the D and J regions of the IgH locus is strongly correlated to H3-K4 methylation (Liu et al. 2007). This association was abolished when RAG2[W453A] was used, indicating that a residue known to be directly involved with H3-K4me3 binding was required for interaction with modified chromatin. Finally, RAG2 proteins with substitutions that abolish H3-K4me3 interaction (Y415A, M443A and W453R) were unable to support robust D to J rearrangement at the IgH locus, confirming that interaction with modified H3 is required for recombination on endogenous loci (Matthews et al. 2007).

Residues within the *nc*RAG2 "hinge" region (388-419) have also been implicated in interaction with core histones (West et al. 2005). *nc*RAG2 was shown to bind to acetylated, methylated, and unmodified core histones, including H2B, H3, and H4. This interaction was isolated to a fragment of *nc*RAG2 spanning amino acids 397-493, including the PHD and a conserved acidic segment in the hinge region upstream. Choosing to focus on this acidic patch, the authors found that substitutions at Y402, N403, D406, or E407 each eliminated core histone binding. In addition, RAG2 with these substitutions was unable to carry out V to DJ recombination on the IgH locus, although it was competent for IgH D to J and kappa locus V to J rearrangement. While this dovetails nicely with the observation that *c*RAG2 knock-in mice have a block at the V to DJ stage, it is not clear why binding to core histones should be required specifically for V to DJ rearrangement and not at other stages of recombination, especially considering the requirement for H3-K4me3 interaction at earlier stages. The authors point out that the acidic patch spanning residues 397-408 bears some resemblance to those found in histone chaperones. One possibility is that RAG2-dependent histone chaperone-like activity is required for the remodeling of chromatin within the IgH V region.

Phosphoinositide binding

Another activity that has been ascribed to PHD modules is phosphotidylinositol phosphate (PIP) binding (Elkin et al. 2005). PIPs can regulate spatial localization and interactions of target proteins and are known to be active in the nucleus at the chromatin level (Martelli et al. 2004). This suggested that PIP binding might affect RAG2 localization or nucleoprotein complex formation. *nc*RAG2 contains a bipartite PIP binding module, including basic residues within the PHD (R464/H468) and residues in the highly conserved extreme C-terminus of the protein (amino acids 521-527) (Elkin et al. 2005). This suggested a functional and possibly physical interaction between these two regions which, unfortunately, could not be investigated structurally due to the poor solubility of the necessary construct. Substitutions within the PHD that reduced but did not abolish PIP binding led to a reduction in the recombination of extrachromosomal substrates. One the other hand, deletion of *nc*RAG2 521-527 completely abolished PIP binding, but lead to a 2- to 3-fold increase in recombination activity.

To explain these data, the authors suggest an equilibrium between two conformations of RAG2: one active for recombination and the other inactive. This was corroborated by buffer-dependent changes in the NMR spectrum of the *nc*RAG2 PHD, suggesting some flexibility in this region. Binding to PIP would favor the inactive configuration; thus the deletion of 521-527 that prevents PIP binding would lead to a hyperactive protein pool. Substitution of residues R464 and H468 is hypothesized to mimic the inactive, PIP-bound configuration, leading to a reduction in recombination activity.

While the most recent work on *nc*RAG2 has focused on H3-K4me3 binding, there is no reason to believe that these activities are mutually exclusive, especially considering that PIP binding involves a portion of *nc*RAG2 that is not required for H3-K4me3 interaction. Future research may demonstrate an important role for PIP in the regulation of recombination.

Regulated RAG2 destruction

V(D)J recombination is restricted to the G0/G1 phase of the cell cycle, the period during which the NHEJ repair pathway for double-stranded DNA breaks is predominant. The primary reason for this is regulated degradation of RAG2 at the G1/S boundary and throughout G2 (Desiderio et al. 1996; Lin and Desiderio 1995). Linkage of RAG2 destruction to the cell cycle relies on the cyclin-dependent kinase cyclinA/Cdk2, which phosphorylates *nc*RAG2 at T490 (Lee and Desiderio 1999). Phosphorylated RAG2 is then ubiquitylated in a manner dependent on the Skp2-SCF complex and degraded by the 26S proteasome (Jiang et al. 2005). Both cyclinA/Cdk2 and Skp2-SCF have been shown to be required for phosphorylation and ubiquitylation, respectively, of RAG2 in cell-free systems, and both are required for RAG2 destruction in living cells. It has also been suggested that phosphorylation of RAG2 may lead to its export to the cytoplasm prior to ubiquitylation and degradation by the 26S proteasome (Mizuta et al. 2002).

Temporal dysregulation of RAG2 has important consequences for recombination. RAG2[T490A] no longer undergoes phosphorylation or cell cycle-dependent destruction. Cells expressing this mutant accumulate recombination intermediates throughout the cell cycle (Jiang et al. 2004), and thymocytes from transgenic RAG2[T490A] animals show an accumulation of aberrant recombination products indicative of repair of V(D)J intermediates independent of the NHEJ pathway (Lee and Desiderio 1999). Thus restriction of recombination to G0/G1 is necessary to avoid some of the potential hazards of V(D)J recombination, including chromosomal instability and aberrant joining. *c*RAG2 lacks T490, so does not undergo cell cycle-dependent regulation. Is cell cycle-dependent regulation of RAG2 essential? Perhaps it is not absolutely essential for V(D)J recombination, but accumulation of chromosomal damage would be expected lead to an increased risk of lymphoid malignancy and reduced overall fitness of the organism. Thus it is not surprising that this regulatory mechanism appears to have been conserved in all vertebrate classes.

Transposition and other nonstandard DNA transactions

The influence of *nc*RAG2 on nonstandard RAG-mediated DNA transactions such as transposition and hybrid joint formation remains controversial. During RAG-mediated transposition, the post-cleavage SEC composed of the two blunt RSS ends held together by RAG1/2 (most often *c*RAG1/2) carries out attack on the phosphodiester backbone of nonspecific target DNA, resulting in covalent attachment of one or both RSS to the target (Agrawal et al. 1998; Hiom et al. 1998). The SEC can also be assembled from a 12/23 pair of blunt RSS ends, bypassing the cleavage step. This reaction occurs with great facility in the test tube, but appears to be very rare in the cell. Only one convincing case of mammalian intracellular RAG-mediated transposition into the chromosome has been documented (Messier et al. 2003), although many attempts have been made to reproduce the activity using both core and full-length (*fl*) RAG1/2. Using a very sensitive episomal assay, Chatterji and colleagues have been able to detect intracellular extra-chromosomal RAG-mediated transposition (Chatterji et al. 2006).

Multiple groups that have examined the effect of *nc*RAG2 on transposition have failed to reach consensus. In the first case, while *c*RAG1/*c*RAG2 was shown to be competent to carry out transposition of blunt RSS ends in a reconstituted system of purified proteins, *c*RAG1/*fl*RAG2 could not, suggesting that *nc*RAG2 was an absolute impediment to the transposition (Swanson et al. 2004). In agreement with this it was further demonstrated that the presence of *nc*RAG2 prevented capture of target DNA (Tsai and Schatz 2003). Conversely, another group using a similar reconstituted system found that *c*RAG1/*fl*RAG2 was competent to carry out transposition of blunt RSS ends at levels slightly elevated relative to *c*RAG1/*c*RAG2. However, *c*RAG1/*fl*RAG2 could not carry out transposition using intact substrates that included coding DNA, suggesting that the presence of coding DNA interfered with capture of the non-specific target (Elkin et al. 2003). Indeed, *c*RAG1/*fl*RAG2 was also deficient in their target capture assay. Yet another group took the approach of isolating intact SECs from cells undergoing recombination of extra-chromosomal substrates and then allowing these SECs to carry out transposition in a cell-free system. SECs including *c*RAG1/*c*RAG2 or *fl*RAG1/*fl*RAG2 were equally competent for transposition under these conditions (Jiang et al. 2004). Strictly speaking, this does not conflict with the observation that the presence of coding DNA interferes with target capture, because coding DNA was not likely to be present in the isolated SECs. Episomal transposition was also shown to be supported by full-length and core RAG proteins (Chatterji et al. 2006). The persistence of SECs in the cell following the release and joining of coding ends coupled with the observation that *nc*RAG2 in SECs formed in cells apparently has no or limited inherent transposition-inhibitory capacity indicates that neither provides an adequate explanation for the very low level of transposition seen in the cell. It should be noted that RAG-dependent transposition of RSS ends has been demonstrated in yeast using both core and full-length RAG1/2 (Clatworthy et al. 2003). Surprisingly, in this system transposition appears to be the preferred method of resolution of

cleaved RSS ends, although both end-joining and transposition occurred at very low rates (1.4×10^{-8} per cell). As a whole, these data suggest that some factor present in vertebrate cells prevents frequent RAG-mediated transposition into the chromosome.

Hybrid and open-and-shut joint formation are two nonstandard reactions in which the RAG proteins mediate attack of the signal ends on the hairpin coding ends. Unlike transposition, hybrid joint formation can be readily reproduced in intracellular systems (Lewis et al. 1988). It has been reported that *fl*RAG1/2 is incompetent for hybrid joint formation in NHEJ-deficient cells (Sekiguchi et al. 2001), but both core and *fl*RAG1/2 support this activity in cell-free systems to similar degrees (Elkin et al. 2003; Swanson et al. 2004). One possibility is that NHEJ factors stabilize the coding ends in the post-cleavage complex, increasing the time available for their attack by the RSS ends. In support of this, *fl*RAG1, but not *c*RAG1, appears to associate with the Ku70/80 NHEJ protein in co-immunoprecipitation assays (Raval et al. 2008).

CONTROVERSIES, CONCLUSIONS, AND FUTURE DIRECTIONS

In the fifteen years since the delineation of the core regions of RAG1 and RAG2, one thing has become clear: while the *nc* regions of these proteins may be "dispensable" for a limited set of biochemical activities, they are in no way "nonessential" for normal immune development. By analogy, eyes and ears are dispensable for digestion, but in many organisms they are essential for actually obtaining food. Likewise, the RAG1/2 *nc* regions donate vital functions such as chromatin binding and cell cycle-specific regulation in the case of *nc*RAG2. Identification of the essential activities of *nc*RAG1 is not as far along, and large stretches of the protein have yet to be fully explored. Continued reductive biochemistry on the modules within the core and *nc* regions coupled with studies of the intact proteins in the whole animal, and every stage in between, will be necessary to ascertain their true contributions to combinatorial immunity.

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