A PHD Finger Motif in the C Terminus of RAG2 Modulates Recombination Activity

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The RAG1 and RAG2 proteins catalyze V(D)J recombination and are essential for generation of the diverse repertoire of antigen receptor genes and effective immune responses. RAG2 is composed of a “core” domain that is required for the recombination reaction and a C-terminal nonessential or “non-core” region. Recent evidence has emerged arguing that the non-core region plays a critical regulatory role in the recombination reaction, and mutations in this region have been identified in patients with immunodeficiencies. Here we present the first structural data for the RAG2 protein, using NMR spectroscopy to demonstrate that the C terminus of RAG2 contains a noncanonical PHD finger. All of the non-core mutations of RAG2 that are implicated in the development of immunodeficiencies are located within the PHD finger, at either zinc-coordinating residues or residues adjacent to an α-helix on the surface of the domain that participates in binding to the signaling molecules, phosphoinositides. Functional analysis of disease and phosphoinositide-binding mutations reveals novel intramolecular interactions within the non-core region and suggests that the PHD finger adopts two distinct states. We propose a model in which the equilibrium between these states modulates recombination activity. Together, these data identify the PHD finger as a novel and functionally important domain of RAG2.

During immune system development, immunoglobulin and T cell receptor genes are assembled from their component gene segments. This process, called V(D)J recombination, is initiated by the lymphoid-specific recombination activating genes 1 and 2 (RAG1 and RAG2) (1, 2). The RAG proteins recognize and bind recombination signal sequences flanking each coding segment and introduce double-strand DNA breaks, which are subsequently resolved into coding joints and signal joints. Processing and joining of the ends require the activity of the ubiquitously expressed proteins from the nonhomologous end joining pathway of DNA repair (reviewed in Ref. 3). V(D)J recombination is critical for proper immune system function; accordingly, mutations in the RAG or nonhomologous end joining proteins result in immunodeficiencies, and inappropriate RAG activity can lead to genomic instability and cancer (4, 5).

RAG2 can be divided into two functionally defined regions, an N-terminal “core” domain (amino acids 1–383) and a C-terminal “non-core” domain (amino acids 384–527) (see Fig. 1A). The core domain is necessary and sufficient for carrying out V(D)J recombination in vitro on exogenous plasmid substrates (6, 7), as well as VDJ cleavage in vitro (8). The non-core domain is dispensable for activity in both of these assays; however, its high conservation throughout evolution suggests it serves critical functions. Indeed, replacement of the endogenous RAG2 gene with only the core domain results in impaired development of B and T cells in mice (9, 10). Moreover, recent studies have implicated the C terminus of RAG2 in the restriction of RAG1/2-mediated transposition (11–13) as well as a nonstandard outcome of V(D)J recombination (14). Finally, mutations within the RAG2 C terminus are linked to the pathogenesis of severe combined immunodeficiency (SCID)1 and Omenn syndrome (OS) (15–18).

Two specific activities encoded within the non-core domain have been identified, a nuclear localization signal and a degradation signal (19, 20). However, the amino acids mutated in SCID and OS do not overlap with the nuclear localization signal or degradation signals. Rather, these mutations are found within a region of the C terminus of RAG2 that was predicted by its primary amino acid sequence to be a PHD finger-like motif (21), although protein-motif predicting programs such as Pfam and Swiss-Prot do not detect a PHD finger within this region under low stringency (data not shown).

The PHD finger motif is present throughout eukaryotic proteomes, most often on chromatin-associated proteins (22).

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1 The abbreviations used are: SCID, severe combined immunodeficiency; OS, Omenn syndrome; PtdIns, phosphoinositides; WT, wild type; αM, α-mannosidase; GST, glutathione S-transferase; NOH, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; IPs, inositol polyphosphates; PIP, phosphatidylinositol phosphate; E3, ubiquitin-protein isopeptide ligase.
Structurally, the PHD finger, like the related FYVE and RING finger modules, belongs to the treble class of zinc-binding domains (23, 24). The FYVE finger is a well characterized PtdInsP-binding module, and RING fingers function as components of E3 ubiquitin ligase enzymes (reviewed in Refs. 25 and 26). Both of these functions have been reported for PHD fingers from different proteins (27, 28), although recent analyses argue that PHD fingers with E3 ubiquitin ligase activity are more likely to be RING finger variants (29, 30). In addition, two recent studies (31, 32) describe PHD finger interactions with nucleosomes.

Structural information about the RAG proteins has long been sought, in hope that it would lend insight into the biochemistry and regulation of V(D)J recombination. In this study, we present the first structural data for the RAG2 protein, demonstrating that the C terminus of RAG2 contains a noncanonical PHD finger. We find that this module directly interacts with PtdInsPs, and mutations that disrupt this binding or cause immunodeficiency can be shown to alter the recombination activity of RAG2. Thus, we identify the PHD finger as a novel and functionally important domain of RAG2.

MATERIALS AND METHODS

NMR Studies and Homology Modeling—Murine Rag2 protein (residues 414–487) in buffer (20 mM Tris, pH 7.2, 50 μM MnSO₄, 13 mM β-mercaptoethanol) was used for the NMR studies. Three pairs of triple resonance experiments HNCO/HN(CA)CO, HNCA/HN(CO)CA, and HNCC/HN(CO)CA were collected by using uniformly ¹⁵N-, ¹³C-, and ²H-labeled protein. The backbone assignments were obtained with the automatic assignment program Ibis (33) and side chain assignments in OS or SCID patients: W453R; 474 N474S; 481 C478Y; 481 H481P. B, family of the 20 lowest energy NMR structures. Cross-eye stereo diagram showing the protein backbone and the zinc-coordinating residues. Red spheres, zinc atoms Zn1 and Zn2.

RESULTS

Solution Structure of the RAG2 C Terminus Reveals the Presence of a PHD Finger Module—To determine directly whether the RAG2 non-core region contains a zinc finger module, the structure of RAG2 from amino acids 414 to 487 (Fig. 1A) was solved using solution NMR spectroscopy (Fig. 1B). The 20 lowest energy structures of 100 calculations were performed with no assumptions about zinc coordination. The NOE contacts and the Cα chemical shifts were sufficient to unambiguously identify Cys-419, Cys-423, Cys-446, Cys-458, and Cys-478 as zinc-coordinating residues (Fig. 1B; Table I). In the RAG2 sequence, cysteines are not positioned to occupy the 4th, 5th, and 8th zinc-binding sites, but a number of histidine residues are appropriately positioned for zinc coordination. The histidine side chain signals in combination with ¹¹¹Cd metal substitution, we identified His-452, His-455, and His-481, family of the 20 lowest energy structures. The protein conformational statistics are shown in Table I. The protein construct encompasses an independently folded protein domain, and the spectral changes induced by EDTA treatment indicate that zinc is required for proper folding and structural stability of the domain (data not shown).

To identify experimentally the zinc-ligating residues, initial structure calculations were performed with no assumptions made about zinc coordination. The NOE contacts and the Cα chemical shifts were sufficient to unambiguously identify Cys-419, Cys-423, Cys-446, Cys-458, and Cys-478 as zinc-coordinating residues (Fig. 1B; Table I). In the RAG2 sequence, cysteines are not positioned to occupy the 4th, 5th, and 8th zinc-binding sites, but a number of histidine residues are appropriately located to be reasonable candidates (21). Using ¹¹¹Cd HSQC analysis of the histidine side chain signals in combination with ¹¹¹Cd metal substitution, we identified His-452, His-455, and
The RAG2 C Terminus Interacts with PtdInsPs—Two functions associated with PHD fingers are PtdInsP binding and E3 ubiquitin ligase activity (27, 28). We have not detected the latter activity for RAG2 (data not shown). To test if RAG2 binds PtdInsPs, protein-lipid blot assays (Fig. 3A) were carried out using in vitro translated full-length RAG2 and core RAG2 (named Core, aa 1–383), which lacks the PHD finger (Fig. 1A). Full-length RAG2, but not Core, bound to the phosphorylated PtdInsP species (Fig. 3B). Similarly, in an independent assay, in vitro translated RAG2, but not Core, bound to PtdInsP-coupled affinity resins (Fig. 3C). These data suggest that RAG2 can bind to PtdInsPs and that the non-core region of RAG2 is required for this activity.

To test directly whether the non-core region binds PtdInsPs, GST-RAG2 C-terminal fusion proteins were assayed for PtdInsP binding in lipid blot (Fig. 3D) or liposome pull-down (Fig. 3E (45)) assays. A C-terminal RAG2 construct containing the PHD finger and a downstream cluster of positively charged residues (RAG2-PHD-CT, aa 414–527) bound PtdInsPs robustly in both assays (Fig. 3, D and E). RAG2-PHD-CT protein bound most strongly to bis-phosphorylated PtdInsPs in the lipid blot overlay assay (Fig. 3D) and to PtdIns(4,5)P$_2$ in the liposome pull-down assay (Fig. 3E). This binding profile is distinct from other PHD fingers tested to date, which characteristically bind preferentially to mono-phosphorylated PtdInsPs (Fig. 3, D and E) (28). Notably, chelation of zinc by EDTA, which disrupts the structure of the RAG2 PHD finger, eliminated PtdInsP binding (Fig. 3D). Unfortunately, the RAG2-PHD-CT aggregates at high protein concentrations, making it unsuitable for NMR structural studies. The RAG2 PHD finger domain alone (RAG2-PHD, aa 414–487), which was used in the structural studies, did not bind to PtdInsPs, and the C-terminal region alone (RAG2-CT, aa 488–527) bound weakly (Fig. 3D). Thus, RAG2-PHD-CT, encompassing both the PHD finger and the C-terminal basic patch, is necessary and sufficient for efficient PtdInsP binding.

Structural Model of RAG2 PHD Finger-PtdInsP Interactions—To gain insight into the structural basis of PtdInsP-RAG2-PHD-CT interactions, the structure of the RAG2 PHD finger was superimposed onto the known structure of the FYVE finger-PtdIns(3)P complex (Fig. 4A) (46). This analysis revealed that a molecular surface of the RAG2 PHD finger, consisting of the L2 α-helix and largely formed by the segment of the polypeptide chain between residues 462 and 474, aligns with the PtdInsP-binding surface of the FYVE finger (Fig. 4A). Considering the importance of positive charge for binding to the acidic head group of PtdInsPs (47), two residues within this region of RAG2, Arg-464 and His-468, are probable participants in PtdInsP recognition (Fig. 4B). Moreover, residues mutated in patients with SCID and OS, Trp-453 and Asn-474, respectively, are adjacent located and predicted to affect this surface area (Fig. 4B). An analogous segment in the ING2 PHD finger contains a basic patch formed by three lysine residues that are necessary for efficient PtdInsP binding by ING2 as well as its pro-apoptotic activity (Fig. 4C) (28). Notably, the contacts formed by this surface alone, however, are not sufficient for PtdInsP binding for either RAG2 or ING2, as both absolutely require residues located C-terminal to the core PHD finger structure (Fig. 4C and see Fig. 3D). Thus, it is likely that the structural basis of RAG2 PHD finger interactions with PtdInsPs is mechanistically analogous to that of other PHD finger proteins.

Functional Analysis of the RAG2 PHD Finger—Although most of the mutations that lead to SCID and OS have been mapped to the core regions of RAG1 and RAG2, several mutations are found within the PHD finger of RAG2 (Fig. 4B),

### Table 1

| Structured residues* | 68 |
|----------------------|----|
| (r.m.s.d.) from mean structure* backbone/heavy atom (Å) | 0.60/1.01 |
| Total NOE distance constraints | 943 |
| Short range (0–1) | 606 |
| Medium range (2–4) | 80 |
| Long range (≥5) | 12 |
| Dihedral angles | 53 |
| Zinc** constraints | 21 |
| Ramachandron plot (%) |
| Most favorable region | 63.8 |
| Additionally allowed region | 34.5 |
| Generously allowed region | 1.5 |
| Disallowed region | 0.2 |

* Residues 415–482 of RAG2. r.m.s.d. indicates root mean square deviation. All values were based on the ensemble of 20 lowest energy structures.

His-481 as zinc-coordinating residues (supplemental Fig. 1). Thus, overall, two zinc ions are bound in the RAG2 C-terminal zinc finger by residues Cys-419, Cys-423, Cys-446, His-452(N2), His-455(N1), Cys-458, Cys-478, and His-481(N1) in the characteristic interleaved topology that is shared by PHD, RING, and FYVE finger modules (Fig. 1B and Fig. 2B).

The $^{113}$Cd substitution experiments revealed that Zn1 is easily substituted during EDTA treatment, whereas Zn2 substitution requires longer EDTA treatment and higher temperatures, indicating that the RAG2 zinc finger has higher affinity for Zn2 than Zn1 (supplemental Fig. 1). Notably, mutations in the Zn2-coordinating residues, Cys-478 and His-481, are found in SCID, suggesting that zinc coordination and integrity of the PHD finger fold is critical for the function of RAG2 (Fig. 1A) (17).

PHD, FYVE, and RING finger modules all contain two zinc ions separated by ~14 Å with two strands of distorted β-sheets connecting the two metal centers (Fig. 2) (23, 41–43). The C-terminal zinc finger of RAG2 has the same topology and clearly belongs in the same structural class. The hydrophobic core and metal coordination geometry in the RAG2 structure contains the characteristic features of the PHD finger module, despite the differences in specific zinc-coordinating residues (Fig. 2A). First, as in other PHD fingers, a histidine ligand of Zn1 (His-455) forms the hydrophobic core of the protein (Fig. 1B). This is confirmed by the slow exchange of the H2-proton, which makes it visible in $^{15}$N HSQC spectra, and numerous NOE contacts of the histidine side chain with other core residues (data not shown). In addition, the coordination of the second zinc is structurally similar to that of other PHD fingers, with two zinc-binding residues (Fig. 2B, C446 and H452) located on both sides of the β-turn (23, 44). This is in contrast to RING fingers, as those residues are shifted toward the second β-strand (43). Finally, the site of the third distorted β-strand C-terminal to the last zinc-binding residues typical of RING fingers is obstructed in RAG2 by the loop between the third and fourth zinc-binding pairs, again similar to what is observed for other PHD finger structures (Fig. 2B). We note that the L1 segment of RAG2 is considerably longer than the L1 segments of other known PHD finger structures (Fig. 2). The L2 segment of the RAG2 zinc finger contains an α-helix (Fig. 2B), which, although not a common feature of PHD fingers, has been observed in a number of PHD finger structures recently deposited in the Protein Data Bank (codes 1WE9, 1WEM, 1WEP, and 1WEV). The packing of this helix is distinct from the packing of the correspondingly located helix in RING fingers (Fig. 2B). Based on these data and consistent with predictions of the primary sequence (21), we conclude that the zinc finger contained within the RAG2 C terminus is a noncanonical PHD finger.
suggesting that integrity of this domain might be critical for normal RAG2 function. To test this hypothesis, a structure-based mutational analysis was carried out to identify PHD finger mutants defective for PtdInsP binding, and the ability of these mutants to execute recombination in vivo was determined.

First, to quantify the relative affinity of RAG2 PHD finger derivatives for PtdInsPs, protein-lipid blot overlays were carried out using lipid blots containing serial dilutions of PtdIns(4,5)P$_2$. As shown in Fig. 5A, at as little as 2.5 pmol, wild-type RAG2-PHD-CT and phospholipase C/H$_{9254}$-PH detect PtdIns(4,5)P$_2$. In contrast, p40-PX, FAPP1-PH, and GST alone bound very weakly or not at all (Fig. 5A). Among the disease-associated mutants, N474S (PHD-CT) interacts with PtdIns(4,5)P$_2$ at a level comparable with wild type, whereas W453R (PHD-CT) and C478F (PHD-CT) displayed a 5-fold reduction in binding, and H481P (PHD-CT) failed to bind (Fig. 5B).

The disease mutants were next tested in a transient V(D)J recombination assay to assess recombination frequencies in vivo. Each recombination assay was performed with full-length RAG1 as the partner protein, using an exogenous substrate to detect either signal joints or coding joints. We first established the recombination activity of full-length RAG2 (WT) in our system; all other constructs were then normalized to WT activity. Consistent with previous reports, Core displayed 20% activity in both signal and coding joint formation compared with WT (Fig. 5C) (6, 48). The N474S mutant carries out recombination at WT levels, consistent with the ability of N474S (PHD-CT) to bind PtdInsPs at WT levels (Fig. 5, B and C). We note that manifestation of the defective phenotype associated with the N474S mutation might only be detectable in recombination at the endogenous loci, or alternatively, it is possible that these patients harbor an additional mutation that contributes to the immunodeficiency. The other three disease mutants all displayed severely reduced recombination frequencies (Fig. 5C). The phenotype of the C478F and H481P mutations is most likely because of disruption in the structure of the protein, as these proteins were poorly expressed compared with WT, W453R, and N474S proteins (Fig. 5D). We note that all the proteins localized to the nucleus, with Core entering the nucleus in a RAG1-dependent manner (data not shown) (20).

Next, we investigated the effects of mutations predicted to disrupt interactions with PtdInsPs. First, a series of truncations eliminating progressively larger sections of the C termini...
nus ending at amino acids 520, 509, and 474 (aa 414–520, 414–509, and 414–474, respectively) were tested for PtdInsP binding. As shown in Fig. 6A, the most extensive truncation (aa 414–474), which eliminates the last two zinc-coordinating residues and almost certainly prevents the PHD finger from folding, not surprisingly fails to bind to PtdInsP. Both the 414–509 and 414–520 truncations, which do not infringe on the core PHD finger sequence, also severely disrupt PtdInsP binding (Fig. 6A). However, the PtdInsP binding activity is not contained at the very C terminus, as a construct containing only the last 7 residues of RAG2 (aa 520–527) fails to bind (Fig. 6A). Thus, the last 7 amino acids of RAG2 are necessary but not sufficient for recognition of PtdInsPs. In this regard, within this region there are two basic residues; however, mutagenesis of these residues to alanines (R523A and R524A, named 2RA-PHD-CT) leads only to a very slight reduction in binding (Fig. 6A).

Analysis of the recombination activity of the truncation mutants is shown in Fig. 6C. The 4747 construct (aa 1–474 of RAG2) had little detectable recombination activity; however, the expression level of this protein is extremely low (Fig. 6E), suggesting that truncation midway through the PHD finger severely compromises stability of the entire protein. Next, we examined constructs 520 (aa 1–520 of RAG2) and 2RA (full-length RAG2 with substitutions R523A and R524A). The 2RA construct had slightly reduced activity relative to WT (Fig. 6C). The 520 construct displayed robust recombination activity, forming coding and signal joints at levels 2–3 times higher than WT (Fig. 6C). This increased activity might be attributable in part to greater stability of the 520 protein versus WT (Fig. 6E;
Regardless, it is evident that disruption of PtdInsP binding does not affect V(D)J recombination on an exogenous substrate and in fact might facilitate this activity (see “Discussion”).

Next, the role of the putative binding surface of the RAG2 PHD finger was investigated (see Fig. 4B, blue α-helix). Substitution of the two basic residues in this region with alanines (R464A and H468A, named 2RH(PHD-CT)) led to a 10-fold reduction in PtdIns(4,5)P₂ binding (Fig. 6B). Analysis of the individual Arg-464 and His-468 mutations revealed an ~5-fold reduction in PtdIns(4,5)P₂ binding for R464A(PHD-CT), relative to wild-type, whereas H468A(PHD-CT), had only a slight reduction.
in binding (Fig. 6B). As a control, a basic residue located on the opposite face of the PHD finger from the α-helix was mutated to alanine (K440A(PHD-CT)) and found to bind PtdIns(4,5)P₂ at wild-type levels (Fig. 6B). Thus, we conclude that Arg-464 and to a lesser extent His-468 participate in PtdInsP recognition. Of note, the recombination activity of 2RH double mutants was severely compromised, forming coding and signal joints at only ~5% of wild-type levels (Fig. 6C). Thus, we conclude that Arg-464 and to a lesser extent His-468 participate in PtdInsP recognition. The indicated constructs were used to determine transient V(D)J recombination activity as in Fig. 6B. Left panel, coding joints. Right panel, signal joints. D, role of Arg-464 and His-468 mutations alone in V(D)J recombination. The indicated constructs were used to determine transient V(D)J recombination activity as in C. Left panel, coding joints. Right panel, signal joints. C and D, * denotes the average of two independent experiments; all other results represent the mean ± S.E. from at least three independent experiments. E, expression of RAG2 and derivative proteins. Western analysis of RAG2 and derivative proteins are as described in Fig. 5D.
and W453R/520, to 2RH and W453R/520). Notably, combining the 2RH mutations with the 2RA mutations (2RH/2RA) had a similar effect (Fig. 6B), suggesting that the 2RA substitution behaves similarly to the 2RH truncation with respect to altering the PtdInsP-binding phenotype of the 2RH mutant, despite having little effect on its own (Fig. 6, A and B). One possible explanation for these data is that a physical interaction, potentially involving bridging by PtdInsPs, occurs between the L2 and CT regions of RAG2 (see “Discussion”).

Based on these data, we hypothesized that the 520 and 2RA mutations might rescue the 2RH mutant recombination phenotype. Indeed combining the 520 or 2RA mutations with 2RH restores coding and signal joint formation back to WT levels (Fig. 6C). Similarly, the 520 and 2RA mutations rescued the single R464A mutation (Fig. 6D). Protein expression and nuclear localization were similar irrespective of the combination of mutations (Fig. 6E; data not shown). In contrast to 2RH, the disease mutants (W453R, C478F, and H481P) had only slightly elevated activity in a 520 background (W453R/520, C478F/520, and H481P/520, Fig. 6C). Again, these proteins all expressed and localized to the nucleus, although C478F/520 and H481P/520 expressed poorly, likely a result of PHD finger misfolding (Fig. 6E; data not shown). Based on these data, we suggest that RAG2 PHD finger mutants fall into one of three phenotypic classes displaying the following: (i) WT levels of PtdInsP binding and recombination activity; (ii) little or no PtdInsP binding and normal to hyper-recombination activity (with the exception of mutants that prevent formation of the PHD finger structure and hence destabilize RAG2); and (iii) intermediate PtdInsP binding (between class I and II) and defective recombination activity in cell culture.

**Role of the PHD Finger in V(D)J Cleavage in Vitro**—It is possible that the third class of mutations structurally destabilize the RAG2 protein, explaining the low recombination activity. Comparison of the 1H HSQC spectra from 15N-labeled samples of wild-type and mutant proteins revealed significant peak shifts localized only to the immediate vicinity of the mutation site (data not shown), indicating that the PHD finger is not misfolded. To rule out an effect on the overall stability of full-length RAG2 protein and to ask whether the class III defects were affecting the DNA cleavage reaction itself versus a different step of V(D)J recombination, recombinant WT and mutant proteins were tested in an in vitro assay for cleavage at a single recombination signal sequences site. As shown in Fig. 7A, 2RH and W453R both cleave substrate at the level of wild-type RAG2. Core, 520, and 520 derivatives all cleave at comparable levels to one another, and do so at a substantially higher level than WT and WT derivatives (Fig. 7A). This difference is likely a manifestation of the increased stability of core and 520 proteins, which results in higher specific activity (Fig. 6E; data not shown). Regardless, these data demonstrate that reduction or elimination of PtdInsP binding does not impact upon RAG2 cleavage activity. Moreover, these data indicate that 2RH and Trp-453 are enzymatically viable, properly folded proteins, arguing that their defect in the transient assay is a consequence of a step distinct from DNA cleavage (see “Discussion”).

**Regulation of In Vitro Transposition by the RAG2 PHD Finger**—It has been shown previously that the C terminus of RAG2 prevents transposition, an activity that might be important for avoiding unwanted genomic instability in developing lymphocytes (11–13). To assess the role of the PHD finger in repressing transposition, we tested in vitro the ability of RAG2 and its derivatives to transpose signal ends starting from either intact or precleaved substrates into an unrelated plasmid (Fig. 7B) (11). On an intact substrate, the transposition event requires the following four steps: (i) cleavage of substrate; (ii) release of the coding ends; (iii) capture of the plasmid target; and (iv) joining of the cleaved signal ends to the plasmid target. The use of a precleaved substrate bypasses the cleavage and release steps, allowing for direct examination of the capture and joining reaction steps (49).

Consistent with previous reports, the Core protein exhibited transposition activity on an intact substrate, whereas WT (and its derivatives) did not (Fig. 7, B and C) (11). The 520, W453R/520, and 2RH/520 proteins had considerably less activity than Core but, nonetheless, still carry out the reaction (Fig. 7, B and C). This might be due to a portion of the inhibitory activity contained within the C terminus being dependent on the last seven amino acids, be a manifestation of the high specific activity of these proteins, or both. As observed previously, wild-type RAG2, although unable to support transposition on an intact substrate, is able to carry out transposition on a precleaved substrate (Fig. 7) (11, 50). In contrast, the 2RH and W453R mutants, which cleave in vitro at levels similar to wild type (Fig. 7A), are compromised in their ability to transpose a precleaved substrate (Fig. 7, B and D). W453R exhibits a very low level of transposition relative to WT, and transposition by 2RH is not detected (Fig. 7D). These data suggest that a func-
tional PHD finger is required during transposition for either target capture, or joining, or both steps. This requirement, however, is bypassed by combining the W453R and 2RH mutations with deletion of the last seven amino acids of RAG2, as on a precleaved substrate, 520, 2RH/520, W453R/520, and Core all display a similar level of activity, which is commensurate with their cleavage activities (Fig. 7B).

DISCUSSION

The C Terminus of RAG2 Contains a PHD Finger—We have presented here the first structural data on RAG2, demonstrating that a region within the non-core C terminus of the protein contains a noncanonical PHD finger (Fig. 1). This finding is in agreement with a study that drew similar conclusions based upon the primary sequence of RAG2 (21). The presence of a PHD finger within RAG2 is consistent with PHD fingers being commonly found on chromatin-associated proteins (22) and with regulation of recombination being fundamentally linked to chromatin structure (reviewed in Ref. 51).

PHD fingers are recognized as clinically important motifs, as mutations within PHD fingers are implicated in tumorigenesis, autoimmune disorders, and other genetic diseases (23, 52, 53). Here we have shown that the four distinct homozygous point mutations found outside of the core region of RAG2 and identified in patients suffering from SCID and OS all fall within the PHD finger of RAG2 (Fig. 4B). Two of these residues, Cys-478 and His-481, are zinc-coordinating residues and are expected to disrupt the PHD finger fold. The other two residues, Trp-453 and Asn-474, are positioned to influence the surface α-helix formed by the L2 segment of the RAG2 PHD finger that participates in interactions with PtdInsPs (Fig. 6). Other examples of disease-related PHD finger mutations located in the vicinity of this L2 surface include a G132N substitution in the ATRX protein implicated in X-linked mental retardation syndrome and a P328L substitution in the AIRE-1 protein implicated in the development of the autoimmune disorder APECED (52, 53). Finally, a basic patch composed of three lysine residues within the analogous segment of the PHD fingers of ING1, ING2, and ACF is required for the PtdInsP binding activity of these motifs (28). We therefore postulate that this surface might be generally utilized as a critical determinant for specifying the binding partners and functions for diverse PHD fingers.

RAG2-PtdInsP Interactions—Recently, the PHD finger of ING2 and a number of other nuclear proteins containing PHD fingers were found to interact with mono-phosphorylated PtdInsPs (28). Our data show that the PHD finger of RAG2 also binds to PtdInsPs, with a preference for bis-phosphorylated PtdInsPs (28). This is consistent with a recent report by our group (29) that the PHD finger of RAG2 of Saccharomyces cerevisiae, using a recently established RAG-dependent recombination system (63).

The Role of the PHD Finger in V(D)J Recombination—We have found that mutations that target the functions or structural integrity of the RAG2 PHD finger display a more severe recombination defect than the Core protein, which lacks the PHD finger (Figs. 5C and 6C). In contrast, mutations in the PHD finger that severely compromise recombination on an exogenous substrate (Figs. 6C and 7A). This phenotype can be entirely reversed by combining the 2RH mutation with deletion or mutation of the last seven amino acids of RAG2 (Fig. 6C). Moreover, deletion of the last seven amino acids restores the ability of both W453R and 2RH to transpose a precleaved substrate (Fig. 7B). Based on these data we hypothesize that a functional, and possibly physical, interaction takes place between the L2 segment of the RAG2 PHD finger and the very C terminus of RAG2, a proposal further supported by the PtdInsP binding data (Figs. 5B and 6A and B). One model to explain these data is that the PHD finger adopts two distinct states, one permissive for recombination and a second inhibitory. Consistent with this idea, changes in buffer pH or high PtdInsP concentrations lead to a small set of peak shifts in the HSQC spectra within the L2 segment of the RAG2 PHD finger, suggesting that the domain is not structurally static (data not shown). We speculate that binding of the PHD finger to a molecular partner, such as PtdInsPs, might modify the equilibrium between these two states. In this context, mutations that eliminate PtdInsP binding, such as the 520 truncation, shift the equilibrium to the permissive state. In contrast, mutations in the PHD finger that...
partially impair PtdInsP binding might adopt a conformation that mimics the bound state and shift the equilibrium toward the inhibitory state. Combining the 2RH mutant with the 520 or the 2RA generates null PtdInsP binders, shifting the equilibrium back toward the permissive state. A similar regulatory mechanism is observed for vinculin and N-WASP, in which PtdIns(4,5)P2 binding shifts the equilibrium between the active and inactive states of these proteins (64, 65). In the case of N-WASP, binding to PtdIns(4,5)P2 is highly cooperative so that homeostatic levels of PtdIns(4,5)P2 do not alter the activity of the protein, and N-WASP is activated only in response to signal-induced changes in PtdIns(4,5)P2 density (65). The observation that the PHD-CT segment of RAG2 alone binds with greater specificity to PtdIns(4,5)P2 (relative to other PtdInsP species) in the context of the molecule being enriched and presented within polymerized liposomes (Fig. 3E) argues that the interaction between RAG2 and PtdIns(4,5)P2 (or possibly a different PtdInsP species) might be cooperative as well (45, 66). This suggests that signal-induced changes of PtdIns(4,5)P2 levels at chromatin might act as a sensitive switch to regulate recombination.

Gene targeted replacement of full-length RAG2 by Core 2RH and 2RA generates null PtdInsP binders, shifting the equilibrium between the active and inactive states of these proteins (64, 65). In the case of N-WASP, binding to PtdIns(4,5)P2 is highly cooperative so that homeostatic levels of PtdIns(4,5)P2 do not alter the activity of the protein, and N-WASP is activated only in response to signal-induced changes in PtdIns(4,5)P2 density (65). The observation that the PHD-CT segment of RAG2 alone binds with greater specificity to PtdIns(4,5)P2 (relative to other PtdInsP species) in the context of the molecule being enriched and presented within polymerized liposomes (Fig. 3E) argues that the interaction between RAG2 and PtdIns(4,5)P2 (or possibly a different PtdInsP species) might be cooperative as well (45, 66). This suggests that signal-induced changes of PtdIns(4,5)P2 levels at chromatin might act as a sensitive switch to regulate recombination.

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