Binding and allosteric transmission of histone H3 Lys-4 trimethylation to the recombinase RAG-1 are separable functions of the RAG-2 plant homeodomain finger

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Antigen receptors are present in the germline as discrete segments that are joined during lymphocyte development by V(D)J recombination (1). V(D)J recombination is initiated by a specialized transposase (2), composed of RAG-1 and RAG-2 subunits, which cleaves DNA at recombination signal sequences (RSSs) that flank the participating gene segments (3). There are two classes of RSSs, termed 12-RSS and 23-RSS, in which heptamer and nonamer elements are separated by spacers of 12 bp or 23 bp; DNA cleavage requires the pairing of a 12-RSS with a 23-RSS (3). Rearrangement is initiated by nicking of DNA by RAG at the junction of each gene segment with its flanking RSS, followed by transesterification to produce double-strand breaks and assembly of the coding sequence by non-homologous end joining (1, 4).

The catalytic and DNA-binding functions reside largely within RAG-1, but RAG-2 is also essential for DNA cleavage, contributing to DNA binding and stabilization of the active site (5). The carboxyl-terminal quarter of RAG-2, comprising residues 388 through 527, is dispensable for DNA cleavage but carries out several regulatory functions, including the binding of RAG-2 to H3K4me3, an epigenetic mark of active chromatin (6–8). V(D)J recombination is preceded by the appearance of noncoding transcripts at the unarranged locus (9, 10) and the deposition of H3K4me3 (6, 7, 11–15), which binds to a plant homeodomain (PHD) finger spanning residues 415 through 487 of mouse RAG-2 (8, 16). Engagement of H3K4me3 promotes recombination in vivo (6, 7) and stimulates coupled cleavage of RSS substrates in vitro (17–19), consistent with allosteric activation. In agreement with this interpretation, binding of H3K4me3 by the RAG-2 PHD finger is associated with changes in the conformational distribution of RAG-1 (20).

The requirement for H3K4me3 recognition can be bypassed by mutations within an inhibitory domain in RAG-2, whose disruption is associated with constitutive increases in RSS binding affinity, catalytic rate, and recombination frequency, mimicking the stimulatory effects of H3K4me3 (17). Association of RAG-1 and RAG-2 with the IgH locus in B cell progenitors depends on recognition of H3K4me3 by the PHD (21, 22); disruption of the inhibitory domain permits association of RAG with the IgH locus in the absence of H3K4me3 binding (23). Thus, the inhibitory domain blocks access of RAG to the IgH locus except when H3K4me3 is engaged by RAG-2.

Taken together these observations suggest the following model for the association of RAG with antigen receptor loci. 1) In the absence of H3K4me3, RAG has low affinity for the RSS. 2) When the RAG-2 PHD finger is engaged by H3K4me3, an allosteric signal is propagated to RAG-1, which acquires high affinity for the RSS. 3) The binding of RAG to a nearby RSS stabilizes its association with the locus. As a test of this model, we sought to identify mutations in the RAG-2 PHD finger that separate binding of H3K4me3 from allosteric activation of RAG-1.

Here, we employ a strategy of phylogenetic substitution to identify amino acid residues within the PHD finger other than those that engage H3K4me3. To test this hypothesis, we sought to...
identify separation-of-function mutations in the RAG-2 PHD finger that impair V(D)J recombination but spare binding to H3K4me3.

**Phylogenetic substitution separates H3K4me3 binding from allosteric activation in vitro**

To decrease the complexity of the search, we employed a phylogenetic approach, reasoning that RAG-2 PHD fingers from species distantly related to the mouse might retain the ability to bind H3K4me3 but be sufficiently different in sequence so that transmission of the allosteric signal to mouse RAG-1 might be impaired. RAG-2 of the brownbanded bamboo shark, *Chiloscyllium punctatum*, is phylogenetically distant from that of the mouse (24). In the interval spanning residues 414 through 485, the RAG-2 PHD finger of *M. musculus* differs from that of *C. punctatum* at 25 positions (Fig. 1A). When mapped onto a crystal structure of the mouse PHD finger (7), these differences largely reside opposite the H3K4me3 binding pocket (Fig. 1B) and are potentially free to participate in an allosteric interface.

The similarity of the *M. musculus* and *C. punctatum* PHD fingers in the region of the H3K4me3 binding site suggested that the shark PHD finger would also be capable of binding H3K4me3. To test this, we constructed a RAG-2 chimera in which the mouse PHD finger was replaced by the PHD finger from *C. punctatum*. Lysates of HEK293T cells expressing the WT murine RAG-2 or the murine-shark RAG-2 chimera were incubated with a bead-bound peptide corresponding to residues 1–21 of histone H3, trimethylated at lysine 4 (H3K4me3). Both WT murine RAG-2 and the mouse-shark RAG-2 chimera bound to the H3K4me3 peptide (Fig. 1C, right, lanes 1 and 3). As expected, a W453A mutation in the H3K4me3 binding pocket of mouse RAG-2 abolished binding to H3K4me3 (Fig. 1C, right, lane 2). In addition, the KMT2D lysine methyltransferase, which carries multiple PHD fingers...
with specificity distinct from that of RAG-2, failed to bind H3K4me3 (Fig. 1C, right, lane 4).

We next asked whether the C. punctatum PHD finger could replace its murine counterpart in an assay for allosteric activation of coupled DNA cleavage by RAG. The version of RAG-1 used in these assays, c1ct, lacks the amino-terminal noncoregion and is more soluble than the WT protein but remains responsive to H3K4me3 (17, 25). Full-length WT mouse RAG-2 and chimeric RAG-2 bearing the C. punctatum PHD finger, both tagged at the amino terminus with MBP, were coexpressed individually with MBP-tagged c1ct, and RAG tetramers were purified by a protocol that removes endogenous H3K4me3 (20). Equivalent amounts of each RAG tetrameric complex were assayed in vitro for coupled cleavage of a radiolabeled 12-RSS substrate in the presence of an unlabeled 23-RSS substrate and increasing amounts of a histone H3-derived peptide containing trimethylated lysine 4 (H3K4me3) or unmethylated lysine 4 (H3K4me0). As reported previously (17, 19, 25), formation of nicked product was stimulated in a dose-dependent manner by H3K4me3 but not by H3K4me0 (Fig. 1D). In contrast, the chimeric version of RAG-2 failed to be stimulated by H3K4me3 (Fig. 1D). Similar results were obtained with independent protein preparations. Taken together, these observations indicated that replacement of the mouse RAG-2 PHD finger with the PHD finger from C. punctatum produces a protein in which the H3K4me3 binding is retained while the ability to support allosteric activation of RAG-1 is lost.

A RAG-2 PHD mutant that binds H3K4me3 without supporting V(D)J recombination in vivo

We went on to test whether the binding of H3K4me3 could be separated from the ability of RAG-2 to support V(D)J recombination in vivo. An initial assay for recombination of an extrachromosomal substrate (17, 23, 26) suggested that the mouse-shark chimeric RAG-2 (PHDPUNC) was impaired in its ability to support V(D)J recombination, as its activity was similar to that of RAG-2(W453A), which cannot bind H3K4me3, and of a chimeric RAG-2 mutant bearing the corresponding mutation, W455A in the C. punctatum PHD finger (Fig. S1).

We proceeded to a more extensive analysis, with the goal of identifying mutations that impair V(D)J recombination while disrupting neither protein expression nor the ability to bind H3K4me3. Cassettes encoding WT RAG-2 and a series of variants, fused at the amino terminus to the FLAG epitope and at the carboxyl terminus to poly-histidine and Myc tags (Fig. 2A), were introduced into the retroviral vector pCLIP2a (27); these included RAG-2(PHDpunc), the chimera bearing the C. punctatum PHD finger in the context of mouse RAG-2. As negative controls, we tested RAG-2(W453A) and RAG-2(PHDpunc,W455A) (Fig. 2A), in which an invariant tryptophan residue involved in H3K4me3 binding was mutated to alanine. We previously showed that disruption of the RAG-2 inhibitory domain permits V(D)J recombination to override the requirement for recognition of H3K4me3 (17, 23). Therefore, it was also of interest to assay RAG-2(D/E352-405A) and RAG-2(D/E352-405A,PHDPUNC) (Fig. 2A), in which the acidic residues within the inhibitory domain were mutated to alanine. We constructed an additional set of mutants in which groups of amino acid residues in the C. punctatum PHD finger were mutated to the corresponding residues in the mouse. Using the three-dimensional structure of the mouse PHD finger as a guide, we assigned these sequence differences to five groups, each of whose residues are closely clustered on the surface opposite the H3K4me3 binding site (Fig. 2, B and C), reasoning that one or more alterations on this surface might disrupt recombination but spare binding to H3K4me3. These spatially clustered mutations, as depicted in Fig. 2B, were termed Group 1 (K418T, S420C, S421P, A422T, and N424D), Group 2 (M425V, N426D, V427I, I429T, E431V, and Y433F), Group 3 (N460D, A462E, S464R, Q465T, Q468H, and Y469L), Group 4 (Q471E, E472G, N473S, and T474N), and Group 5 (F445Y, S448H, F477Y, and Y482V), with the numbering following that of the mouse sequence.

To assay recombination quantitatively, we employed a fluorescent assay for recombination of an integrated substrate (28). Retroviral constructs encoding the RAG-2 variants described in Fig. 2A, as well as constructs encoding the clustered shark-to-mouse mutants, were used to infect the cell line R2K3, a RAG-2-deficient, Abelson murine leukemia virus-transformed B progenitor (28). R2K3 contains an integrated substrate for V(D)J recombination whose inversional rearrangement results in the expression of GFP. Following transduction of RAG-2, recombination is induced by STI-571; rearrangement of the integrated substrate is detected by flow cytometry. Cells transduced with WT RAG-2 exhibited increasing levels of GFP fluorescence, approaching a recombination frequency of 80%, over a period of at least 96 h following arrest with STI-571 (a representative analysis at 96 h is shown in Fig. 3A; the results of three technical replicate assays at 48 h and 96 h are shown in Fig. 3B). As expected, recombination was impaired by the W453A mutation, which disrupts binding of H3K4me3 (Fig. 3, A and B). We also observed little recombination in cells transduced with the RAG-2(PHDpunc) chimera or with RAG-2(PHDpunc,W455A) (Fig. 3, A and B). Notably, the RAG-2(D/E352-405A,PHDPUNC) chimera, in which the inhibitory domain is neutralized, supported recombination to a level near to that of WT RAG-2 or RAG-2(D/E352-405A) (Fig. 3, A and B, IDNEUT-PHDpunc), indicating that the chimeric protein can interact functionally with RAG-1 to support recombination when the requirement for H3K4me3 binding is bypassed. Among the back-mutated mutants, RAG-2(PHDpunc,Group1) and RAG-2(PHDpunc,Group4) showed sharply diminished recombination activity with respect to WT RAG-2, while RAG-2(PHDpunc,Group2), RAG-2(PHDpunc,Group3), and RAG-2(PHDpunc,Group5) supported recombination at frequencies about 38–75% that of WT (Fig. 3, A and B).

Because any impairment of recombination could result from decreased protein accumulation, we examined expression of each of the RAG-2 variants at 96 h after arrest by STI-571, with the goal of identifying mutant proteins that are robustly expressed but fail to support efficient V(D)J recombination. Accumulation of RAG-2(PHDpunc) was reduced to less than one tenth that of WT RAG-2 (Fig. 3C; Fig. S2) and could not be
rescued by overexpression of RAG-1 in a cotransfection assay (Fig. S3). Of the additional mutant proteins that exhibited impaired recombination in the R2K3 assay, only RAG-2 (PHDPUNC, Group1) was expressed at a level similar to those of RAG-2 variants that retained recombination activity (Fig. 3C; Fig. S2). Thus, the Group 1 mutation permitted near-normal accumulation of the RAG-2(PHDPUNC) chimeric protein but was associated with a large reduction in recombination activity.

To confirm the effects of the RAG-2 mutations described above on V(D)J recombination, the R2K3 rearrangement assay was performed in biological triplicate, with each biological assay consisting of three technical replicates (Fig. 3D). Of note, cells transduced with RAG-2(PHDPUNC,Group1) exhibited a large and statistically significant decrease in recombination frequency, compared with cells transduced with WT RAG-2 or RAG-2(PHDPUNC,Group2) (Fig. 3D, Group 1), while neutralization of the inhibitory domain robustly conferred recombination activity on the RAG-2(PHDPUNC) chimera (Fig. 3D, IDNEUT-PHDPUNC).

**A back-mutation from shark to mouse sequence in the RAG-2 PHD that restores V(D)J recombination activity**

We considered that the impairment of recombination activity observed for the RAG-2(PHDPUNC,Group1) mutant might result from disruption of intramolecular interactions that propagate the allosteric signal initiated by the binding of RAG-2 to H3K4me3. To test this interpretation, we asked 1) whether the defect in recombination observed for RAG-2(PHDPUNC,Group1) could be reversed by an additional back-mutation within the C. punctatum PHD finger; and 2) whether RAG-2(PHDPUNC,Group1) retains the ability to bind H3K4me3. Because RAG-2(PHDPUNC,Group2) robustly supported V(D)J recombination, the Group 2 back-mutation was an attractive candidate for rescue of RAG-2(PHDPUNC,Group1). We observed that a mouse-shark RAG-2 chimera bearing combined Group 1 and Group 2 mutations exhibited a robust increase in recombination frequency, relative to the Group 1 mutant (Fig. 4, A and B, compare Group 1 to Groups 1 + 2). In this assay, the expression levels of RAG-2(PHDPUNC,Groups1 + 2), RAG-2(PHDPUNC,
Group1), and WT RAG-2 differed by about 2-fold or less (Fig. 3C). Taken together, the results described above indicate that the Group 1 mutation enhances accumulation of the RAG-2 (PHDPUNC) chimera without permitting recombination and that additional back-mutation of the Group 2 cluster confers recombination activity on the Group 1 mutant. Consistent results were obtained with a similar recombination assay in which RAG-2 variants were transduced using the pCST virus (Fig. S4).

To establish that RAG-2(PHDPUNC,Group1) is indeed a separation-of-function mutant, we tested its ability to bind H3K4me3. WT mouse RAG-2, RAG-2(W453A), RAG-2(PHDPUNC), RAG-2(PHDPUNC,Group1), RAG-2(PHDPUNC,Group2), and RAG-2(PHDPUNC,Group1 + 2) were expressed as MBP fusions in HEK293 cells. Lysates were incubated with beads coated with the H3K4me3 peptide or the corresponding unmethylated peptide (H3K4me0). Bead-bound protein was detected by immunoblotting for MBP. As expected, WT RAG-2 was capable of binding to H3K4me3 but not to H3K4me0, while RAG-2(W453A) bound neither peptide (Fig. 4D). Consistent with results presented above, RAG-2(PHDPUNC) was able to bind H3K4me3. Importantly, RAG-2(PHDPUNC,Group1), RAG-2(PHDPUNC,Group2), and RAG-2(PHDPUNC,Group1 + 2) each exhibited binding to the H3K4me3 peptide but not to H3K4me0 (Fig. 4D). Taken together, the properties of the RAG-2(PHDPUNC,Group1) mutant indicate that the ability of the RAG-2 PHD finger to bind H3K4me3 can be separated from its ability to support V(D)J recombination.

Discussion

Binding of H3K4me3 to the RAG-2 PHD finger alters the conformation of RAG-1, and this is accompanied by enhancements of affinity for substrate and catalytic rate (6, 7, 17, 19, 20). These observations have suggested an allosteric model for
activation of RAG. According to such a model, binding of H3K4me3 by the RAG-2 PHD finger would be necessary but insufficient for activation of RAG-1. The observations described here provide additional support for an allosteric model by demonstrating that activation of RAG-1 requires one or more functions of the RAG-2 PHD finger in addition to engagement of H3K4me3.

An initial indication that H3K4me3 binding is insufficient for activation of RAG was provided by RAG-2(PHDPUNC), which retains the ability to bind H3K4me3 but was unresponsive to allosteric activation in vitro by an H3K4me3 peptide under conditions in which equivalent amounts of WT RAG and chimeric mutant were compared. Although this observation is consistent with allostery, we considered a more trivial explanation for loss of activity, namely, that RAG-2(PHDPUNC) might have undergone a structural alteration that renders it unable to support DNA cleavage, for example, by disrupting a association with RAG-1. This is rendered unlikely by the fact that disruption of the inhibitory domain restores the ability of the RAG-2(PHDPUNC) chimera to support recombination. Therefore, we favor the interpretation that the PHD finger of C. punctatum is unable to transmit an activating allosteric signal to mouse RAG-1 upon binding of H3K4me3.

Although RAG-2(PHDPUNC) satisfied biochemical criteria for a separation-of-function mutant, its poor expression in vivo precluded our assessing its function in V(D)J recombination. Through systematic reversion of RAG-2(PHDPUNC) residues specific to C. punctatum to those of the mouse, we identified three clustered mutations, RAG-2(PHDPUNC,Group2), RAG-2(PHDPUNC,Group3), and RAG-2(PHDPUNC,Group5), which were able to confer recombination activity. Because each of these mutations also restored protein accumulation to an amount similar to that of WT RAG-2, their stimulatory effect on recombination might have been a consequence of increased expression. Alternatively or in addition, these mutations might have effected an increase in the affinity of the PHD finger for H3K4me3 or enhanced the efficiency with which information regarding the engagement of H3K4me3 is transmitted to RAG-1. In this regard, it should be noted that the clustered Group 3 and Group 5 mutations reside near the H3K4me3 binding site.

The RAG-2(PHDPUNC,Group1) mutant, like RAG-2(PHDPUNC,Group2), RAG-2(PHDPUNC,Group3), and RAG-2(PHDPUNC,Group5),...
Relay of an allosteric signal through the RAG-2 PHD

was expressed robustly in vivo. Unlike RAG-2(PHD_{PHDPUNC,Group2}),
RAG-2(PHD_{PHDPUNC,Group3}), and RAG-2(PHD_{PHDPUNC,Group5}), RAG-2
(PHD_{PHDPUNC,Group1}) exhibited a large and significant reduction in
the ability to support V(D)J recombination, even though it
 retained the ability to bind H3K4me3. Thus, with respect to
recombination, RAG-2(PHD_{PHDPUNC,Group1}) represents a separa-
tion-of-function mutant. We considered the possibility that
RAG-2(PHD_{PHDPUNC,Group1}) might have suffered an overall debili-
tating structural lesion, but we consider this unlikely because
RAG-2(PHD_{PHDPUNC,Groups1 + 2}), in which the Group 1 and Group
2 mutations are combined, exhibited full function. Lastly, we con-
sidered that RAG-2(PHD_{PHDPUNC,Group1}) might have exhibited a
loss of function because of an inability to interact with an essen-
tial factor other than RAG-1. This is unlikely because allosteric
activation of coupled RSS cleavage does not require components
other than RAG-1, RAG-2, and H3K4me3 peptide.

Available RAG structures do not include the PHD finger and
therefore do not provide information regarding its interactions
(5, 29, 30). A recent study (31) provides evidence for an interac-
tion between RAG-1 and the dispensable region of RAG-2, but
the structural basis for this interaction remains unexplored. The
Group 2 mutation, which converted six residues in the C. puncta-
tatum PHD finger (M425, N426, V427, I429, E431, and Y433) to
the corresponding residues of the mouse was able to restore
function to the RAG-2(PHD_{PHDPUNC,Group1}) mutant in an assay
for V(D)J recombination. The ability of the Group 2 mutation to
rescue RAG-2(PHD_{PHDPUNC,Group1}) is not likely to be the result of
effects on protein accumulation or H3K4me3 binding, although
the latter possibility is not excluded by the available data. This
result strongly suggests that, in the mouse RAG-2 PHD finger,
one or more of the residues V425, D426, I429, T429, V431, and
F433 participates in the relief of autoinhibition exerted by the
RAG-2 inhibitory domain, either through transmission of the
H3K4me3 allosteric signal or in some other, as yet undefined,
way. Because the mutational strategy employed here was re-
stricted to amino acid sequence differences between M. muscu-
lus and C. punctatum, it remains likely that other residues con-
tribute to the activation of RAG-1 in response to H3K4me3.

Although the PHD finger of RAG-2 from C. punctatum binds
H3K4me3, it has not, to our knowledge, been determined
whether the C. punctatum RAG orthologue is subject to allo-
steric activation by H3K4me3. Alignment of RAG-2 from M. muscu-
lus and C. punctatum does, however, reveal 43% sequence identity within the interval from residue 352 through
residue 405 of the mouse sequence, corresponding to the inhibi-
tory domain (23). Moreover, the corresponding C. punctatum
sequence, like that of the mouse, is highly acidic, suggesting the
presence of an autoinhibitory function within RAG-2 of C.
punctatum that may be relieved by H3K4me3.

The PHD finger of human RAG-2 is a frequent target of
mutations associated with primary immunodeficiency diseases
(32). Disease-associated missense mutations have been identi-
ﬁed at 13 sites within the PHD; mutations at 8 of these residues
have been found in patients with Omenn syndrome, a primary
immunodeficiency disorder characterized by pathogenic oligo-
clonal T cell expansion and profound B lymphopenia (32–35).
The remaining mutations are associated with severe combined
immunodeficiency, atypical severe combined immunodeficiency,
Relay of an allosteric signal through the RAG-2 PHD

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Abbreviations—The abbreviations used are: V(D)J, variable-diversity-joining; RAG, recombination-activating gene protein; MBP, maltose-binding protein; PHD, plant homeodomain; RSS, recombination signal sequence; ANOVA, analysis of variance; H3K4me3, histone H3 trimethylated at lysine 4; H3K4me0, histone H3 with unmethylated lysine 4.

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