The RAG1 and RAG2 proteins together constitute the nuclease that initiates the assembly of immunoglobulin and T cell receptor genes in a reaction known as V(D)J recombination. RAG1 plays a central role in recognition of the recombination signal sequence (RSS) by the RAG1/2 complex. To investigate the parameters governing the RAG1-RSS interaction, the murine core RAG1 protein (amino acids 377–1008) fused to a short Strep tag has been purified to homogeneity from bacteria. The Strep-RAG1 (StrRAG1) protein exists as a dimer at a wide range of protein concentrations (25–500 mM) in the absence of DNA and binds with reasonably high affinity and specificity (apparent $K_D = 41$ nM) to the RSS. Both electrophoretic mobility shift assays and polarization anisotropy experiments indicate that only a single StrRAG1-DNA species exists in solution. Anisotropy decay measured by frequency domain spectroscopy suggests that the complex contains a dimer of StrRAG1 bound to a single DNA molecule. Using measurements of protein intrinsic fluorescence and circular dichroism, we demonstrate that StrRAG1 undergoes a major conformational change upon binding the RSS. Steady-state fluorescence and acrylamide quenching studies reveal that this conformational change is associated with a repositioning of intrinsic protein fluorophores from a hydrophobic to a solvent-exposed environment. RSS-induced conformational changes of StrRAG1 may influence the interaction of RAG1 with RAG2 and synaptic complex formation.

The genes encoding the variable domains of immunoglobulins or T cell receptors are generated during lymphocyte differentiation by a somatic recombination reaction known as V(D)J recombination (1). The reaction is initiated by DNA double strand breaks created at the junction between two coding segments (termed V, D, or J) and their flanking recombination signal sequences (RSSes). Cleavage is followed by a complex repair process that results in imprecise joining of the two coding segments and typically precise joining of the two RSSs. The RSSs consist of two conserved sequence elements, the heptamer (consensus 5'-CACAGTG-3') and the nonamer (consensus 5'-ACAAAAACC-3'), separated by a poorly conserved spacer sequence of either 12 or 23 base pairs. Efficient recombination occurs only between a 12-RSS and a 23-RSS, a phenomenon known as the 12/23 rule (for review, see Ref. 2). Recognition of 12/23-RSSs and concerted cleavage at the RSS-coding sequence border is performed by a complex of the RAG1 and RAG2 proteins (for reviews, see Ref. 3 and 4), the lymphoid-specific products of the recombination-activating genes RAG1 and RAG2 (5, 6). Binding and cleavage of DNA by the RAG1-RAG2 complex is facilitated by the ubiquitously expressed architectural DNA-binding proteins HMG1 and -2 (7, 8).

Deletion mutagenesis has established the minimal "core" domains of murine RAG1 (residues 384–1008 of the 1040 aa RAG1 protein; Fig. 1A) and RAG2 (residues 1–383 of the 527 aa RAG2 protein) required for recombination activity in transfected nonlymphoid cell lines (9–12). Most biochemical studies of V(D)J recombination have been performed with the core RAG proteins because of their enhanced solubility compared with the full-length proteins.

Surface plasmon resonance (13) and in vivo one-hybrid (14) experiments demonstrated specific recognition of the nonamer by the RAG1 nonamer binding domain and a less significant contribution of the heptamer to binding. In the one-hybrid experiments, RAG2 only modestly enhanced RAG1 DNA binding (14), implying that RAG1 by itself was capable of significant, sequence-specific DNA binding. Subsequently, numerous studies making use of electrophoretic mobility shift assays (EMSA) and, in most cases RAG proteins fused to large N-terminal tags (glutathione S-transferase [GST] or maltose-binding protein [MBP]), led to the identification of a stable RAG1-RAG2-RSS complex termed the SC (for signal complex) (15–18). These studies also indicated that RAG1 alone binds the RSS with low specificity and that RAG2 substantially enhances the affinity and specificity of the interaction. Such findings have led to the suggestion that the RAG1-RSS interaction is unlikely to be biologically significant in the absence of RAG2 (15). Only weak, nonspecific DNA binding by RAG2 alone has been reported (17), but recent evidence supports a direct interaction between RAG2 and DNA in the RAG1-RAG2-RSS complex (19). Footprinting experiments (15, 20, 21) reveal that RAG1 generates protection over the nonamer and the flanking 

RAG1-DNA Binding in V(D)J Recombination
SPECIFICITY AND DNA-INDUCED CONFORMATIONAL CHANGES REVEALED BY FLUORESCENCE AND CD SPECTROSCOPY*

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DNA-induced Conformational Change in RAG1

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EXPERIMENTAL PROCEDURES

DNA Oligonucleotides—Unlabeled and fluorescently labeled deoxy-
ligonucleotides were synthesized and high performance liquid chroma-
tography-purified by Integrated DNA Technologies Inc. (Coralville, IA).

DNA-induced Conformational Change in RAG1

The stoichiometry of the RAG proteins in the SC is contro-
versial. Some experiments indicated the presence of a dimer of
RAG1 (18, 22, 23, 24), whereas others argue for the presence of
three or four RAG1 subunits (25, 26). RAG2 is present in the
SC either as a monomer (18) or as a dimer (23, 26). The nonamer binding domain of RAG1 is thought to contact the
nonamer, whereas a central domain of RAG1 (aa 528–760) has
been shown to display some specificity for the heptamer (27).
In addition, a C-terminal domain of RAG1 has been shown to
be cross-link to the coding flank (28). Recent experiments indicate
a division of labor between RAG1 monomers in the SC; one
contacts the nonamer, whereas another engages the heptamer
at the site of cleavage (25, 29).

We currently do not know the equilibrium constant that
governs the association of RAG1 monomers into dimers. Fur-
thermore, since most DNA binding studies have been per-
formed by EMSA, we do not know the equilibrium constants
governing reactions involving RAG1 alone or RAG1 in conjunc-
tion with RAG2 and HMG proteins in forming complexes with
the RSS in solution. The equilibrium dissociation binding con-
stant for the interaction of a MBP-RAG1 core (aa 384–1008)
fusion protein with 12- or 23-RSS oligonucleotides was deter-
mined by EMSA to be close to 100 nM (22). In this study,
however, the MBP-RAG1 fusion protein was capable of forming
several protein-DNA complexes with various protein stoicho-
imetries and various DNA binding affinities, and the deter-
mined dissociation constant reflected the overall binding with-
out specification of the individual components of reaction.

We have now used fluorescence spectroscopy and a RAG1
protein lacking a bulky tag to study the RAG1-RSS interaction
in solution, thereby overcoming some of the limitations inher-
ent in the EMSA methodology and any complications caused by
a large fusion partner. We find that the RAG1-RSS interaction
is of higher affinity and specificity than previously reported. In
addition, we document a substantial conformational change in
RAG1 induced by interaction with the RSS. Based on these
findings, we propose that the RAG1-RSS interaction may play
an important role in vivo, especially in light of the observation
that for a portion of the cell cycle, developing lymphocytes
express RAG1 in the virtual absence of RAG2 protein (30).

DNA-induced Conformational Change in RAG1

The 12-RSS substrate was made by annealing (top strand) 5′-GTCG-
CCACAGTCATACAGGTGACAAAACCCCTGCGAG-3′ with its
complement. The nonspecific DNA substrate was made by annealing (top strand) 5′-GTGCACTGGCCATCTACAGCTAGCGGCGCCTCG-
CAG-3′ with its complement. The fluorescent fluorophore was attached
at the 5′ end of the top strands using a 5′-fluorescein phosphoramidite
that attaches 6-carboxyfluorescein to the oligonucleotide via a C6 chain
linker. Annealing of oligonucleotides to generate double-stranded DNA
was performed in binding buffer (BB); 10 mM Tris-HCl (pH 7.5), 50 mM
NaCl, 5 mM MgCl2 by heating the complementary oligonucleotides
mixed in equimolar amounts for 5 min at 95 °C followed by slow cooling
to room temperature.

Expression and Purification of Strep-RAG1 (StrRAG1)—Murine core
RAG1 was expressed as a Strep-tag II fusion in the context of the
pASK-IBA5 vector (Sigma Genosys). The catalytic core of murine RAG1
from aa 377–1008 was amplified using primers that created an Ndel site
at the 5′ end of the gene and a XhoI site at the 3′ end. These PCR
products were subcloned into the corresponding sites in pASK-IBA5,
placing the Strep tag at the N terminus of the protein.

The fusion protein was expressed in the BL21 strain of Escherichia
coli. Expression cultures were started by adding 20 ml of overnight

culture per 1 liter final culture volume and grown at 35 °C with rapid
shaking (~200 rpm) until the A600 was ~1. Expression was induced by
the addition of anhydrotetracycline to a final concentration of ~1 μM
and ZnCl2 to a final concentration of 0.1 mM. Cultures were grown for
an additional 12–16 h, at which point the A600 was usually ~5 absorb-
ance units. Pelleted bacteria were resuspended in ice-cold lysis buffer
(20 mM Tris (pH 8.0), 100 mM NaCl, 0.5 mM EDTA, 5 mM 2-mercap-
toethanol, 2 mM MnCl2, 10% glycerol, and protease inhibitor mixture
(phenylmethylsulfonfluoride, pepstatin A, aprotinin, leupeptin).
Cells were lysed by sonication on ice, and debris and insoluble material
were pelleted by ultracentrifugation at 26,000 rpm for 1 h at 4 °C in a
SW-41 rotor (Beckman). Cleared lysate was added to Fast-Flow Q-
Sepharose (Amersham Biosciences) 50–75 ml bed volume column pre-
equilibrated in the lysis buffer before the addition of lysate. After
loading, the column was washed with two bed volumes of lysis buffer
containing 200 mM NaCl, and RAG1 was eluted with 3 bed volumes of
lysate buffer containing 500 mM NaCl. Q-Sepharose elution fractions
were applied to a 7–10 ml bed volume Streptactin-Sepharose column
(Sigma Genosys) pre-equilibrated in lysis buffer containing 500 mM
NaCl, and after washing with lysis buffer containing 500 mM salt,
Strep-RAG1 was eluted in lysis buffer (500 mM salt) containing 2.5 mM
desthiobiotin. The eluted Strep-RAG1 protein was typically >90–95% pure.
Aggregated Strep-RAG1 was separated from dimers and other
oligomers with a Superdex 200HR column (Amersham Biosciences).

Fractions containing the dimeric protein were dialyzed against BB.
MBP-RAG1 core (aa 384–1008) and glutathione S-transferase-RAG2
(22–1–383) protein expression and purification were performed as de-
scribed in (22, 31).

Coupled Cleavage Assay—Coupled cleavage reactions (50-μl final
volume) contained 10 ng of body-labeled substrate containing both a
12-RSS and a 23-RSS, 100 ng of each RAG protein, 30 ng of HMG2, and
5 μg MgCl2 and were incubated for 2 h at 37 °C using buffer conditions
described previously (32). Reaction products were resolved on a 4% native
Tis borate EDTA polyacrylamide gel.

EMSA—To create the EMSA probe, 2 μM 12-RSS double-stranded
oligonucleotide DNA was 5′-end labeled using T4 polynucleotide kinase
(New England Biolabs) and [γ-32P]ATP (9000 Ci/mmol) (PerkinElmer
Life Sciences) and purified on a 5% native polyacrylamide gel. Strep
or MBP-RAG1 was incubated with radioactively labeled 12-RSS oligo-
nucleotide in the presence or absence of unlabeled 12-RSS or nonspe-
cific DNA in BB supplemented with 10 ng/ml heparin and 0.5% glycerol
at 25 °C for 20 min, and samples were resolved on 4% native polyacryl-
amide gels in 0.5 × Tris borate (45 mM Tris (pH 8.9), 45 mM borate) at
room temperature. For better separation of the multimeric species
formed by MBP-RAG1, a discontinuous 3.5–8% polyacrylamide gel was
used. Gels were dried and quantitated using a PhosphorImager (Mo-
lecular Dynamics). The fraction bound at saturation, maximal fraction
bound, Fmax, is 1, is set constant, whereas we expressed the normalized
fraction bound as the ratio between the fraction bound at each data set
and the actual constant value of the fraction bound at saturation. The
data were fit to a Hill binding isotherm with n = 2.

normalized fraction bound = Fmax[StrRAG1] [Kapp] + [StrRAG1]

(1–Eq. 1)

[StrRAG1] represents the concentration of free protein expressed as
monomeric protein.

Steady-state Fluorescence Measurements—The StrRAG1 intrinsic
emission fluorescence spectra were recorded using a SLM-8000 L format
spectrophotometer equipped with a 750-watt Xenon arc lamp. All
fluorescence measurements were performed in 100-μl quartz cuvettes
(Brand, Covaderno, CA) at 25 °C constant temperature, ther-
by a circulating water bath. The StrRAG1 intrinsic emission
fluorescence spectra were recorded with an excitation wavelength of 280 nm
using an 8-nm band pass for both the monochromator and the emission long
pass. All fluorescence emission spectra were recorded between 300 and
420 nm using 1-nm steps and 2-s integration times. Dilution of StrRAG1 samples was done in ice-cold BB buffer in the
presence of 0.2-μm sterile filters (Gelman Laboratory) followed by a 10-min
incubation at 25 °C. For static experiments, mixing of StrRAG1 with DNA
or acrylamide was done inside the quartz cuvette followed by a 10-min
incubation at 25 °C constant temperature before recording. Each spec-
trum was corrected (33) for background, photobleaching (5–15%),
and any contribution to a contrast relative to a control sample of
protein was incubated in parallel with a corresponding volume of buffer.
Photobleaching was always reversible, indicating that no photo prod-
ucts are generated in the reaction. The fluorescence emission intensity of
all spectra were expressed as a ratio relative to a standard rhodamine
reference excited with the same source and whose emission output was a constant ~46,500. If sample absorption at excitation wavelength exceeded 0.001, inner filter corrections were also applied according to Lakowics (34). For the time course experiment shown in Fig. 7b, DNA was mixed with protein rapidly with a long capillary tip with the cuvette in the holder and an automatic shutter opened immediately for recording. The emission at 327 nm was recorded at 1-s intervals.

**Circular Dichroism Measurements**—Circular dichroism spectra either of DNA or DNA incubated with StrRAG1 was recorded using a JASCO J715 spectropolarimeter. The buffer used for incubation was 10 mM NaCl, 10 mM KCl, 5 mM MgCl2. The spectra were recorded at 25 °C between 200 and 300 nm. For analysis of the spectra of DNA and StrRAG1 mixtures, the separately recorded DNA spectra were subtracted, and then the spectra were converted to molar ellipticity based upon the molar concentration of StrRAG1 present in each sample.

**Fluorescence Data Analysis**—For every set of anisotropy data, a minimum of three experiments was performed under identical conditions, and the data points shown are average values. Each averaged data point was considered only if the sample S.D. was less than 5–7% with respect to the calculated mean. The fitting of averaged data points was performed using a typical Hill binding isotherm (36),

\[
 f = \frac{[L]_{\text{tot}} - n_{\text{obs}}[R]_{\text{tot}}}{K_D}
\]

(Eq. 3)

The real solutions \( f = [R(L)]/[R]_{\text{tot}} \) of Equation 3 were substituted into the fluorescence anisotropy expression to be fitted to,

\[
 r_{\text{obs}} = r_0 + f(r_{\text{sat}} - r_0)
\]

(Eq. 4)

The best fit for StrRAG1 binding curve to fluorescein-labeled 12-RSS DNA was obtained with the Hill coefficient \( n = 1.6 \). The solution calculations and fitting were performed using Maple 7.0 and Origin 6.0 software.

For experiments in which tryptophan anisotropy was monitored while adding unlabeled 12-RSS, because StrRAG1 quantum yield changes with DNA binding we calculated the correction factor \( R = F/F_0 \), where \( F_0 \) is the relative quantum yield (derived from the integral value of fluorescence corrected spectra) of free StrRAG1, and \( F \) is the relative quantum yield of DNA bound StrRAG1 at saturation.

**RESULTS**

**Catalytically Active, Dimeric RAG1 Protein Lacking a Bulky Tag**—In our study, we used a bacterially expressed and purified murine RAG1 protein (aa 377–1008) fused to its N terminus to a short (8 aa) Strep-II tag (Sigma Genosys, Inc.) that allows affinity purification with Streptactin-coupled Sepharose (Fig. 1a). Three purification steps result in a StrRAG1 protein of greater than 98% purity (Fig. 1b). The elution profile from the final gel filtration column reveals a prominent peak at the position expected for a StrRAG1 dimer (compare Fig. 2, a and c; the predicted molecular mass of the monomer is 72 kDa). When the purified dimeric protein was reanalyzed on the gel filtration column, only the dimer peak was observed, indicating

comprising cooperative interaction factors, which reflects the ligand concentration at half-maximal anisotropy. To fit our data values, Equation 2 was written as a function of \([L]_{\text{tot}}, \) total concentration of the ligand species, and \([R]_{\text{tot}}, \) total concentration of the reporter species in solution.

\[
 f_1 = \frac{[L]_{\text{tot}} - n_{\text{obs}}[R]_{\text{tot}}}{K_D}
\]

where \( n_{\text{obs}} \) is the observed anisotropy of the reporter species, \( r_{\text{obs}} \) is the basal anisotropy of the reporter species in the absence of ligand, \( r_{\text{sat}} \) is the maximal anisotropy reached at saturation, \([L] \) is the free ligand concentration varied in the reaction, expressed as monomeric species, \( n \) is the Hill coefficient expressing the degree of cooperative ligand binding to the reporter species, and \( K_D \) is the apparent dissociation constant.
that the protein remains stably associated in dimeric form (Fig. 2b). The intrinsic polarization anisotropy of the purified StrRAG1 at concentrations from 25 to 500 nM is constant, consistent with the existence of protein as a dimer at all concentrations within this range (data not shown). In a standard coupled cleavage assay, the StrRAG1 protein was approximately as active as highly purified, dimeric MBP-core RAG1 (aa 384–1008) (Fig. 1c).

By EMSA, StrRAG1 Binds Specifically to the RSS, Forming a Single Protein-DNA Complex—We first tested the DNA binding ability of StrRAG1 by EMSA. Increasing amounts of StrRAG1 (or for comparison, MBP-RAG1) were incubated with a constant amount of labeled DNA bearing a consensus 12-RSS sequence. The binding buffer used throughout this study contains a moderate salt concentration (50 mM NaCl and 5 mM MgCl₂) and was chosen to minimize nonspecific RAG1-DNA interactions (13, 14).

In Solution, StrRAG1 Binds with Higher Affinity and Specificity to the 12-RSS Than Detected by EMSA—To circumvent the disadvantages associated with EMSAs, we used fluorescence polarization to investigate interactions between StrRAG1 and DNA in solution. Fluorescence anisotropy provides an indirect measure of the rotational diffusion of molecules in solution, which is in turn dependent on their size and shape (larger molecules have lower tumbling rates and have higher anisotropy values) (34). The first experiments made use of double-stranded 12-RSS DNA or nonspecific oligonucleotides labeled at the 5′ end of one strand with fluorescein (12-RSS-Fl, nonspecific-DNA-Fl), which allowed measurement of the anisotropy of the DNA molecules (excitation wavelength 492 nm, emission recorded at 520 nm). 12-RSS-Fl or nonspecific-DNA-DNA-Fl there is a reduced, non-saturable linear decrease in intrinsic anisotropy (Fig. 4b). The curve for 12-RSS-Fl has a sigmoidal shape with increasing anisotropy values that reach saturation around 100 nM StrRAG1 (monomeric protein concentration) and an increase of anisotropy of 90–100% over the value in the absence of protein. The best fit of the data points (correlation = 96%; sum of squares = 0.65 × 10⁻⁴) was achieved using a Hill binding isotherm with n = 1.8 (Equations 3 and 4), yielding an apparent Kₐ = 41 ± 8 nM (reflecting monomeric protein concentration). This value for Kₐ corresponds to an affinity of interaction higher than that determined by EMSA experiments. The increase in 12-RSS DNA anisotropy observed in this experiment is due solely to the formation of protein-DNA complexes, which increase the molecular size reported by the fluorophore attached to the DNA. In Fig. 4b the same anisotropy data are represented as a function of molar ratio of StrRAG1:12-RSS, which should yield an inflection point indicating binding stoichiometry (37). The inflection point observed indicates dimeric protein binding to 12-RSS DNA. In the case of nonspecific-DNA-DNA-Fl there is a reduced, non-saturable lin-
ear increase in anisotropy, corresponding to nonspecific binding of StrRAG1 to DNA. The specificity of the StrRAG1-DNA interaction was also examined by anisotropy competition experiments. First, 50 nM 12-RSS-Fl was incubated with 100 nM StrRAG1, and then either specific 12-RSS or nonspecific unlabeled DNA was added in increasing amounts to the mixture (Fig. 4c). Similar to the results of EMSA competition experiments, only the 12-RSS was capable of dissociating the 12-RSS-Fl/StrRAG1 complex and reducing the anisotropy to values characteristic of 12-RSS-Fl DNA alone. Even a 100-fold excess of nonspecific DNA resulted in only a slight reduction (12–15%) in the anisotropy of the complex.

In conclusion, polarization anisotropy experiments using fluorescently labeled 12-RSS show that StrRAG1 binds relatively tightly (K_D = 41 ± 8 nM) and specifically to DNA in solution. The inflection point in our anisotropy data strongly suggest a molar ratio of protein to DNA of 2:1 in the StrRAG1-RSS complexes.

**DNA Rotational Reorientation Times**—To further investigate the stoichiometry of StrRAG1 in protein-DNA complexes in solution, we used frequency domain fluorescence spectroscopy (FD). Frequency domain fluorescence measures the fast changes in either phase or modulation that occur in the fluorescent light emitted a short time after the fluorophore is excited with a coherent source of light (34). These changes

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**Fig. 2.** StrRAG1 exists in solution as a dimer. a, gel filtration chromatography (Superdex 200HR) profile from the last purification step. Approximately 70% of the protein is present in dimeric form. b, fractions 13 and 14 corresponding to the dimeric protein in were combined, and after concentration, the protein was subjected again to gel filtration chromatography. Essentially all of the protein remains in dimeric form. c, gel filtration chromatogram of protein calibration markers separated on the same Superdex 200 HR column. IgG, goat purified immunoglobulin G; BSA, bovine serum albumin; Ribo A, ribonuclease A.
FIG. 3. By EMSA, StrRAG1 binds specifically to the 12-RSS, forming a single protein-DNA species. 

a, EMSA using 75 nM labeled 12-RSS double-stranded oligonucleotide (40-mer) and increasing amounts of StrRAG1, as indicated above the lanes. The inset shows the saturation curve obtained from quantifying the bands corresponding to free probe and 12-RSS/StrRAG1 complex. The fraction bound represents the fraction of 12-RSS/StrRAG1 complex formed normalized relative to the maximal binding obtained at saturation. M, molecular mass standards. 

b, EMSA using 75 nM 12-RSS probe and increasing amounts of MBP-RAG1. A discontinuous 3.5%/8% polyacrylamide gel was used, as indicated at the right. 

c, competitive EMSA to assess specificity. 200 nM StrRAG1 was incubated with 75 nM labeled 12-RSS in the absence (lane 2) or presence of increasing concentrations of unlabeled 12-RSS (lanes 3–9) or nonspecific (nsp) DNA (lanes 10–14). Labeled probe and competitor DNA were added simultaneously to the StrRAG1 protein.
allow direct calculation of the fluorophore life-time \(\tau\) and the rotational reorientation time \(\theta\) (when the emitted fluorescence comes through a vertically oriented polarizer), which is the inverse value of the rotational diffusion coefficient of the molecule (tumbling rate of molecule).

We first measured the lifetime of the fluorescein fluorophore attached to the 12-RSS or non-specific DNA (Table I) and found that it was not significantly changed by the addition of StrRAG1 or HMG2 (a non-specific DNA binder) from the standard 3.6-4.1 ns value reported for fluorescein alone in pH 7.5 aqueous solutions (42). These important control measurements demonstrate that neither DNA bases nor protein amino acid side groups interact with fluorescein or significantly change the fluorophore environment when these proteins interact with the DNA.

We then used frequency domain differential phase and polarized modulation ratio measurements to determine the time-resolved anisotropy decay of the fluorescein-labeled 12-RSS or non-specific DNA in the presence or absence of StrRAG1 or HMG2 (Table I). Fitting of the anisotropy decay curves was performed using a double exponential equation (35, 43) (see “Experimental Procedures”). In this equation, one exponent describes the rotational diffusion of the fluorophore with respect to the whole molecule (local rotational reorientation time, \(\theta_{\text{local}}\)), and the other describes the global rotation of the macromolecules (global rotational reorientation time, \(\theta_{\text{global}}\)). In all cases, \(\theta_{\text{local}}\) is almost unchanged from the 0.35-0.4-ns value, confirming that the fluorophore does not interact with the bases or the proteins during protein-DNA complex formation. \(\theta_{\text{global}}\) values change considerably upon protein binding, indicating significant changes in the size of 12-RSS-FI DNA in complexes with StrRAG1 under conditions of partial binding (50 nM protein) or near binding saturation (100 nM StrRAG1) (44, 45). Importantly, much lower global rotational reorientation times are reported for non-specific DNA in the presence of StrRAG1, either at 50 or 100 nM protein concentrations, confirming the lower representation of protein-DNA species that form non-specifically in solution in our assay conditions (Table I). From the calculated \(K_p\), one can estimate that more than 85% of the 50 nM 12-RSS DNA is present in protein-DNA complexes in the mixture with 100 nM StrRAG1 (saturation conditions). Hence, the global rotation reorientation time obtained in this reaction predominantly represents that of the protein-DNA complex. Using the rotation reorientation time \(\theta_{\text{Global}} = 48.03 \pm 10.05\text{ ns}\) obtained under saturation binding conditions and the original Perrin equation (Equation 7; see “Experimental Procedures”), we can estimate the size of the StrRAG1:12-RSS complex to be between \(\sim 175\) kDa (using \(h = 0\) ml/g, an anhydrous sphere) and \(120\) kDa (using \(h = 0.4\) ml/g, the maximal degree of hydration) (46, 47). Given the apparent molecular masses of the protein (72 kDa) and 12-RSS-FI DNA (31 kDa), we conclude that StrRAG1 is most likely present as a dimer in these complexes.

**Tryptophan Fluorescence Anisotropy of StrRAG1 Increases in the Presence of the 12-RSS**—We wanted to monitor by fluorescence anisotropy the effect of adding DNA to StrRAG1 having as a reference the protein present in solution. This set of polarization anisotropy experiments took advantage of the intrinsic fluorophores in the StrRAG1 protein (primarily its 6 tryptophan residues) to serve as the fluorescent reporters (excitation wavelength 295 nm, emission recorded at 327 nm). Hence, these experiments measured changes in the size of the protein complexes whether or not they were bound to DNA. StrRAG1 (250 nM) was incubated with increasing concentrations of unlabeled 12-RSS, and tryptophan anisotropy was measured (Fig. 5). With the 12-RSS, anisotropy increased abruptly and reached a saturation value about 20% greater than that of the protein alone. The data fit a rectangular hyperbola, with an apparent \(K_p = 16.5 \pm 7\text{ ns}\). This apparent \(K_p\) value reflects the 12-RSS DNA concentration present in solution at half-maximal increase in anisotropy. Because the protein binds as a dimer, this \(K_p\) value is expected to be

![Fig. 4. StrRAG1 binds as a dimer to the 12-RSS with \(K_p = 41\text{ nM}\). a, fluorescence polarization anisotropy of 50 nM 12-RSS-FI fluorescein labeled DNA (black squares) incubated in solution with increasing concentrations of StrRAG1. Each data point represents an average of five individual determinations. Using a Hill equation with \(n = 1.8\) generates a fit with 96% correlation and a sum of squares of \(0.65 \times 10^{-4}\) (Equations 3 and 4, see “Experimental Procedures”). Open triangles show results obtained with fluorescein-labeled non-specific DNA (nsp-DN AFl) incubated with the same concentrations of StrRAG1. Each data point represents an average of three determinations. b, determination of the stoichiometry of StrRAG1-DNA binding by fluorescence anisotropy. The anisotropy data of panel a are plotted against the molar ratio [StrRAG1]/[12-RSS-FI] present in the reaction (calculated for monomeric StrRAG1). The intersection of the linear-fitting of the slope and plateau yields a molar ratio of two StrRAG1 monomers per 12-RSS-FI DNA molecule (37). c, the 12-RSS-StrRAG1 complex is resistant to competition with non-specific DNA. 50 nM 12-RSS-FI was incubated with 100 nM StrRAG1, and after DNA-protein complex formation, either unlabeled 12-RSS (filled squares) or non-specific DNA (open triangles) were added in increasing concentrations. Each data point represents the average of three individual determinations. The arrow points the level of anisotropy of free 12-RSS-FI. The inset describes that the fluorophore does not interact with the bases or the proteins during protein-DNA complex formation. \(\theta_{\text{local}}\) values change considerably upon protein binding, indicating significant changes in the size of 12-RSS-FI DNA in complexes with StrRAG1 under conditions of partial binding (50 nM protein) or near binding saturation (100 nM StrRAG1) (44, 45). Importantly, much lower global rotational reorientation times are reported for non-specific DNA in the presence of StrRAG1, either at 50 or 100 nM protein concentrations, confirming the lower representation of protein-DNA species that form non-specifically in solution in our assay conditions (Table I). From the calculated \(K_p\), one can estimate that more than 85% of the 50 nM 12-RSS DNA is present in protein-DNA complexes in the mixture with 100 nM StrRAG1 (saturation conditions). Hence, the global rotation reorientation time obtained in this reaction predominantly represents that of the protein-DNA complex. Using the rotation reorientation time \(\theta_{\text{Global}} = 48.03 \pm 10.05\text{ ns}\) obtained under saturation binding conditions and the original Perrin equation (Equation 7; see “Experimental Procedures”), we can estimate the size of the StrRAG1:12-RSS complex to be between \(\sim 175\) kDa (using \(h = 0\) ml/g, an anhydrous sphere) and \(120\) kDa (using \(h = 0.4\) ml/g, the maximal degree of hydration) (46, 47). Given the apparent molecular masses of the protein (72 kDa) and 12-RSS-FI DNA (31 kDa), we conclude that StrRAG1 is most likely present as a dimer in these complexes.
DNA-induced Conformational Change in RAG1

Fluorescein life-time ($\tau$) measured by FD spectroscopy either for DNA alone or DNA in the presence of various concentrations of StrRAG1 or HMG2. *The short life time for 12-RSS-Fl reflects the presence of free, uncoupled fluorescein. The FD anisotropy decay traces were fitted by Global analysis to a double exponential decay (35) from which the local rotation reorientation time $\theta_{local}$ (corresponding to the fluorophore) and global rotation reorientation time $\theta_{global}$ (corresponding to the entire molecule or ensemble of molecules) could be obtained.

| DNA            | Protein          | Fluorescein lifetime, $\tau$ | $f_1$ | Local rotational reorientation time, $\theta_{local}$ | $f_2$ | Global rotational reorientation time, $\theta_{global}$ |
|----------------|------------------|------------------------------|-------|------------------------------------------------------|-------|-------------------------------------------------------|
| 50 nM 12RSS-FI | 50 nM StrRAG1    | 3.97                         | 0.91  | 0.33 ± 0.05                                          | 0.13  | 11.46 ± 0.95                                          |
| 50 nM 12RSS-FI | 50 nM StrRAG1    | 3.97                         | 0.91  | 0.33 ± 0.05                                          | 0.13  | 11.46 ± 0.95                                          |
| 50 nM nonspecific DNA-FI | 50 nM StrRAG1 | 3.98                         | 0.88  | 0.57 ± 0.01                                          | 0.12  | 8.9 ± 0.17                                            |
| 50 nM 12RSS-FI | 100 nM StrRAG1   | 4.03                         | 0.87  | 0.46 ± 0.02                                          | 0.13  | 48.03 ± 10.05                                         |
| 50 nM nonspecific DNA-FI | 100 nM StrRAG1 | 3.98                         | 0.88  | 0.41 ± 0.02                                          | 0.12  | 14.87 ± 1.11                                          |
| 50 nM 12RSS-FI | 15 nM HMG2       | 4.01                         | 0.75  | 0.41 ± 0.02                                          | 0.25  | 17.81 ± 2.29                                          |
| 50 nM nonspecific DNA-FI | 15 nM HMG2 | 4.20                         | 0.82  | 0.48 ± 0.02                                          | 0.18  | 16.60 ± 2.25                                          |

Table I. Lifetime and rotation reorientation time values measured by frequency domain spectroscopy

Fig. 5. Intrinsic tryptophan fluorescence polarization anisotropy of 250 nM StrRAG1 incubated with increasing amounts of 12-RSS DNA (dark diamonds) or nonspecific DNA (open triangles). Each data point represents the average of five individual determinations. The 12-RSS data points were fit with a rectangular hyperbola ($y = 5 \times 10^{-4}, R^2 = 0.89,$ yielding an apparent $K_D = 15.5 \pm 7$ nM, taking into account the quantum yield corrections for the bound fraction (see Equation 6, "Experimental Procedures"), whereas the nonspecific (nsp)-DNA data were fit to a linear function.

approximately one-half of the $K_D$ value obtained from the data points in Fig. 4a, which reflects the concentration of monomeric StrRAG1 present in the reaction under similar conditions. The increase in tryptophan anisotropy is predominantly due to an increase in the molecular size of a significant population of StrRAG1 molecules in the presence of 12-RSS DNA. This increase can be due either to formation of protein-DNA complexes or to formation of higher order protein-protein oligomers not associated with DNA. In the presence of nonspecific DNA, there is an increase in anisotropy to levels 5–6% above the value for the free protein, and the increase is linear and nonsaturable.

Binding of StrRAG1 to DNA Causes Major Changes in the Protein Intrinsic Fluorescence Spectrum—We next wanted to ask whether DNA alters the conformation of the StrRAG1 protein. First, we tested if the intrinsic emission fluorescence spectrum (excitation at 280 nm, emission at 350 nm) of StrRAG1 protein is altered by DNA. Upon incubation of 100 nM StrRAG1 with increasing amounts of 12-RSS oligonucleotide, a drastic decrease in emission fluorescence intensity (fluorescence quenching) occurred, saturating at 50–60% of the fluorescence in the absence of DNA (Fig. 6a). As with the increase in tryptophan polarization anisotropy (Fig. 5), this decrease in fluorescence caused by 12-RSS DNA can be fit to a rectangular hyperbola (Fig. 6c), but the effect does not quantitatively parallel the DNA binding phenomenon. It is important to note that since the quenching effect occurs at concentrations of DNA lower than those expected to yield a significant amount of protein-DNA complexes, it is possible that interaction with the 12-RSS induces a change in protein configuration (see below).

Next, we performed kinetic experiments to determine whether quenching and binding occur simultaneously. First, 50 nM 12-RSS labeled with fluorescein was mixed with 50 nM StrRAG1, and the change in DNA anisotropy was measured with time (Fig. 7a). 10–15 s after mixing, anisotropy reached levels expected from the static equilibrium experiment (Fig. 4a) and then remained unchanged for 13 min. This indicates that protein-DNA complexes are formed very rapidly. In the second experiment, 50 nM StrRAG1 was incubated with 10 nM 12-RSS or with nonspecific DNA, and intrinsic StrRAG1 fluorescence was measured with time. The 12-RSS induced a complex, slow multiphasic quenching of StrRAG1 fluorescence, quite distinct from that induced by nonspecific DNA. The first phase, which lasts ~200 s, was characterized by a substantial (17%) decrease in fluorescence. In the second phase, lasting between 200 and 700 s, fluorescence increased transiently, perhaps because of transient changes in fluorophore environment caused by protein conformational changes. Surprisingly, StrRAG1 intrinsic fluorescence quenching continued even after 800 s but at a much reduced rate. Although the kinetic behavior of StrRAG1 intrinsic fluorescence will require further study, it is clear that DNA binding (Fig. 7a) occurs much more rapidly than fluorescence quenching (Fig. 7b). We conclude that fluorescence quenching predominantly reflects conformational changes induced by the 12-RSS DNA.

Acrylamide Quenching Indicates That 12-RSS Binding Causes StrRAG1 Fluorophores to Become More Exposed to Solvent—To investigate the mechanism by which 12-RSS DNA acts as a quencher of StrRAG1 intrinsic fluorescence, we tested the effect of acrylamide on the StrRAG1 fluorescence in the presence or absence of DNA. Acrylamide is a small insoluble molecule that quenches both tryptophan and tyrosine fluores-
cence and has access to regions of a protein where a macromolecule like DNA does not. Therefore, adding acrylamide after protein-DNA complex formation tests whether the intrinsic protein fluorophores become more exposed to the solvent interface (in the case of a conformational change) or less exposed to solvent molecules (if they are involved in stable complexes either with DNA bases or with other surrounding amino acids) relative to the protein fluorophores in the absence of DNA. Increasing concentrations of acrylamide were added to 600 nM StrRAG1 in the absence or presence of 150 nM specific 12-RSS or nonspecific DNA, and protein intrinsic emission fluorescence was measured. In Fig. 8, the abscissa represents the ratio between the original sample fluorescence in the absence of acrylamide (F₀) and the sample fluorescence (F) at the indicated concentration of acrylamide on the ordinate (Stern-Volmer plot) (38). In Table II, we present the values of the quenching constants derived from fitting our data to the quadratic Equation 8 (see “Experimental Procedures” and “Discussion”). The upward curvature of the Stern-Volmer plots indicates the combined effect of dynamic and static quenching (see “Experimental Procedures”). The slope of the curve, however, is increased by the presence of DNA (and more by 12-RSS than nonspecific DNA), indicating that DNA causes some of the amino acid fluorophores to become more accessible to the quencher, as reflected in the increased values of their dynamic quenching constants (Table II). This result is also consistent with the red shift of the spectra that occurs upon the addition of DNA. StrRAG1 has a wealth of fluorophores (6 Trp and 20 Tyr residues), and it is, therefore, difficult to address where in StrRAG1 quenching occurs. In summary, these experiments indicate an important role for dynamic (solvent mediated) quenching of intrinsic fluorophores of StrRAG1 upon interaction with DNA, and that this effect is stronger with specific than nonspecific DNA.

Circular Dichroism Provides Further Support for a DNA-induced Conformational Change in StrRAG1—We then asked if the 12-RSS-induced conformational change in StrRAG1 observed by fluorescence could also be detected by circular dichroism (CD). Fig. 9 presents the close and far UV CD spectra of 500 nM StrRAG1 protein alone or in the pres-
ence of increasing amounts of 12-RSS DNA. The prominent minima peaks at 210 and 222 nm are indicative of significant \textit{a} helical content in StrRAG1 (48). Upon 12-RSS addition, the molar ellipticity at both of these characteristic wavelengths decreases dramatically, saturating at a DNA concentration of 50–60 nM (Fig. 9b). When nonspecific DNA was used, the spectral changes observed were smaller (Fig. 8, c and d; compare b with d).

Together, the StrRAG1 fluorescence and CD spectral changes induced by 12-RSS DNA lead to the conclusion that RSS sequences induce major conformational changes in StrRAG1. In addition, because these changes are much greater...
with 12-RSS than nonspecific DNA, sequence-specific interactions apparently underlie much of the observed changes.

**DISCUSSION**

**RSS Binding by RAG1 in the Absence of RAG2**—In an early study using an *in vivo* one-hybrid assay, core RAG1 was found to interact specifically with a tandem array of eight 12-RSS elements, and RAG2 appeared to enhance binding only modestly (14). Based on this and other findings, it was suggested that RSS recognition might be a two-step process consisting of RAG1 binding followed by recruitment of RAG2 (13, 14). The results of subsequent *in vitro* experiments provided at least two types of arguments against the general validity of this model. First, it was found that the initial rate of DNA cleavage could be enhanced by preincubating MBP-RAG1 with RAG2 but not by preincubating MBP-RAG1 with the DNA (49). Second, studies relying primarily on EMSA methodology and RAG1 proteins with a bulky tag suggested that the RAG1-RSS interaction was not sufficiently specific to be physiologically relevant (see the Introduction). The only study to investigate the equilibrium binding of highly purified, dimeric RAG1 core protein used EMSA methodology and documented an approximate 10-fold specificity for the RSS and a reasonably high affinity (150 nM) (22). However, as confirmed here, the MBP-RAG1 core protein used in those experiments forms three species with the RSS in which the protein is present in various oligomeric states.

We show here that a RAG1 core protein with only a short eight amino acid tag is soluble and forms stable dimers in solution in the absence of DNA. Both EMSA and solution DNA binding assays demonstrate that this protein binds the RSS as a dimer and that it does so specifically and with moderately high affinity (in solution, apparent $K_d = 10^2$ nM). Furthermore, the complex that forms with the 12-RSS is relatively resistant to competition with nonspecific DNA (Fig. 6c) and by a number of different measures is distinct from that formed upon interaction with nonspecific DNA. These findings lead us to reconsider the relevance of the two-step model for RSS recognition noted above.

In developing lymphocytes in the G1 phase of the cell cycle, RAG1 and RAG2 are co-expressed, with RAG2 apparently in molar excess (50). In the S, G2, and M phases of the cell cycle, however, RAG2 is rendered unstable by phosphorylation by a cyclin-dependent kinase (51, 52), and RAG2 levels drop dramatically, whereas RAG1 levels are almost unchanged (30). We would like to propose that in S, G2, and M phases, RAG1 can bind to RSSs in the absence of RAG2. Furthermore, we propose that as these cells complete mitosis and enter G1, and RAG2 levels begin to rise, RAG2 can be recruited to RSSs by pre-bound RAG1, creating a catalytically competent complex. This does not exclude the possibility that some and perhaps the majority of RSS recognition is performed by RAG1-RAG2 complexes. Instead, our results lead to the idea that a pattern of RAG1-RSS interactions laid down in S/G2/M may be relevant to the subsequent targeting of the recombination reaction.

**A DNA-induced Conformational Change in RAG1**—It has been proposed previously that RAG2 can induce a conformational change in RAG1 that facilitates binding of RAG1 to the RSS and perhaps also the appropriate folding of the RAG1 DDE active site (3, 18, 27). Until the experiments reported here, however, there have been no studies of conformational changes in the RAG proteins either induced by one another or by DNA. We have been able to study the interactions of StrRAG1 with DNA in solution by monitoring protein intrinsic fluorescence, circular dichroism spectra, and fluorescence polarization. These studies would likely have been considerably less informative if RAG1 had been attached to bulky MBP or glutathione S-transferase tags due to the spectral contributions of the fusion partners.

Two pieces of evidence support the conclusion that RSS DNA induces a conformational change in RAG1. The addition of RSS DNA to StrRAG1 results in (i) strong quenching of intrinsic StrRAG1 protein fluorescence (Fig. 6) and (ii) a dramatic alteration in the CD spectrum of the protein (Fig. 9). We consider each of these in turn. The decrease in fluorescence intensity and red shift of $\lambda_{max}$ observed when StrRAG1 interacts with 12-RSS DNA could have two, not mutually exclusive, explanations.

(i) Dynamic quenching could occur if some of the main fluorophores (Trp and Tyr residues) of the protein undergo a change in environment from the hydrophobic core to the solvent interface of the macromolecule, where they are subject to dynamic quenching by solvent molecules. The red shift of $\lambda_{max}$ when StrRAG1 was incubated with 12-RSS DNA is in itself an indication that the dielectric constant $\epsilon$ of the environment of some of the protein fluorophores increases, consistent with the red shift reported for the indole ring in cyclohexane versus water (53). This can only be caused by a conformational change in the protein structure. Such changes would also increase accessibility of the protein fluorophores to the small molecule quencher acrylamide, and hence, one would expect stronger acrylamide quenching for strRAG1 plus DNA than for strRAG1 alone. This is exactly what is observed in Fig. 8.

(ii) Static quenching could occur if some of the internal fluorophores of StrRAG1 became involved in direct complexes with amines of DNA bases. Alternatively, the DNA molecule could be an indirect static quencher if upon binding to StrRAG1, it induces a protein conformational change that causes charged amino acid residues to form direct complexes with Trp or Tyr fluorophores (the charge transfer that occurs in such complexes forbids the decay of the excited state and prevents photon emission). Direct static quenching by DNA does not necessarily involve a protein conformational change. In these cases, stronger acrylamide quenching should be observed for StrRAG1 alone than for the 12-RSS-StrRAG1 complex since fluorophores involved in static quenching cannot be quenched further by acrylamide. This scenario, however, is in conflict with the data that we present in Fig. 8. This reasoning was applied in assigning the static and dynamic acrylamide quenching ($K_s, K_d$) values presented in Table II, which were simply derived as solutions of the quadratic Equation 8 (see “Experimental Procedures”).

The kinetics of 12-RSS-induced quenching of StrRAG1 fluorescence shows a complex pattern (Fig. 7b) and occurs considerably more slowly than protein-DNA complex formation. Quantitatively, the 12-RSS specifically and efficiently quenches StrRAG1 intrinsic emission fluorescence, with the effect reaching saturation at DNA concentrations where only a fraction of the protein is engaged in forming StrRAG1-DNA complexes (Fig. 6c).

Together, our observations are best explained by a model in which dimers that dissociate from the DNA either retain the newly acquired conformation or further modify their configur-
tion (as the transient increase in fluorescence in Fig. 7b suggests) and, hence, are as susceptible or even more susceptible to solvent quenching than those bound to DNA. Therefore, it is possible that the 12-RSS acts as a "catalyst" to induce major conformational changes in StrRAG1 whether bound to the DNA or not.

We note that the oligomerization state of other DNA-binding proteins such as λ repressor (54) and human immunodeficiency virus integrase (55) has been shown to be influenced by the presence of DNA. In the case of human immunodeficiency virus integrase, time-resolved anisotropy measurements of its tryptophan rotation reorientation times show that integrase exists in various oligomeric states in solution, and Mg²⁺ and DNA cause dissociation of the tetrameric form to the monomeric form that is found in complex with DNA (55, 56).

The addition of 12-RSS DNA and to a lesser extent nonspecific DNA results in a dramatic change in the CD spectrum of StrRAG1 (Fig. 9). A possible explanation for the DNA-induced decrease in both 209- and 222-nm peaks (indicative of α helicity (57)) is the movement of the peptide groups from an orientation almost parallel to the helix axis towards a tilted configuration with carbonyl groups pointing outwards, due to a drastic change in the protein backbone. Such a reorientation of the peptide groups has been predicted to decrease the amplitude of long wavelength CD peaks of proteins with α helix content (58).

The stoichiometry of pre-cleavage RAG1-RAG2-RSS complexes is controversial, with two reports suggesting that these complexes contain more than two (presumably four) monomers of RAG1 (25, 26), and another suggesting that they contain only a single dimer of RAG1 (24). If the former model is correct, then since DNA-bound StrRAG1 dimers show no propensity to associate with other StrRAG1 dimers, it is likely that stable association of two dimers of the RAG1 core with an RSS requires RAG2. The situation might be different for the full-length RAG1 protein, which contains an additional zinc-dependent dimerization domain (59).

In conclusion, we demonstrate that the core RAG1 protein containing a small epitope tag binds the RSS as a dimer and undergoes a conformational change upon doing so. Our results provide a starting point for future studies of possible RAG2-induced conformational changes in RAG1.

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Fig. 9. Circular dichroism (CD) spectra displaying molar ellipticity (Mol. θ) of StrRAG1 versus wavelength (nm) in the absence or presence of DNA. a, CD spectra of 500 nM StrRAG1 in the absence or presence of increasing concentrations of 12-RSS DNA. b, molar ellipticity of StrRAG1 as a function of 12-RSS concentration, recorded at 210 nm (open circles), 222 nm (filled squares), and 245 nm (open triangles). c, CD spectra of 500 nM StrRAG1 in the absence or presence of increasing concentrations of nonspecific DNA. d, molar ellipticity of StrRAG1 as a function of nonspecific DNA concentration, recorded at 210 nm (open circles), 222 nm (filled squares), and 245 nm (open triangles).
RAG1-DNA Binding in V(D)J Recombination: SPECIFICITY AND DNA-INDUCED CONFORMATIONAL CHANGES REVEALED BY FLUORESCENCE AND CD SPECTROSCOPY
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