The Role of the SAP Motif in Promoting Holliday Junction Binding and Resolution by SpCCE1*

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Holliday junctions are four-way branched DNA structures that are formed during recombination and by replication fork regression. Their processing depends on helicases that catalyze junction branch migration, and endonucleases that resolve the junction into nicked linear DNAs. Here we have investigated the role of a DNA binding motif called SAP in binding and resolving Holliday junctions by the fission yeast mitochondrial resolvase SpCCE1. Mutation or partial/complete deletion of the SAP motif dramatically impairs the ability of SpCCE1 to resolve Holliday junctions in a heterologous in vivo system. These mutant proteins retain the ability to recognize the junction structure and to distort it upon binding. However, once formed the mutant protein-junction complexes are relatively unstable and dissociate much faster than wild-type complexes. We show that binding stability is necessary for efficient junction resolution, and that this may be due in part to a requirement for maintaining the junction in an open conformation so that it can branch migrate to cleavable sites.

Homologous recombination plays a prime role in DNA metabolism. It repairs double-strand breaks and stalled/collapsed replication forks, guides chromosome segregation at meiosis, and generates genetic diversity. A key intermediate of homologous recombination is the four-way DNA (Holliday) junction, which forms as a consequence of strand exchange catalyzed by the RecA/Rad51 family of proteins (1). Holliday junctions also form by regression of stalled replication forks (2–5). Once formed, the Holliday junction is subject to branch migration through regions of homology and/or cleavage. Such processing is pivotal both for determining the outcome of the recombination reaction and for enabling replication to restart at stalled forks.

Holliday junctions are cleaved by a ubiquitous but diverse group of metal ion-dependent endonucleases called the Holliday junction resolvases (for reviews, see Refs. 6 and 7). Resolvases are typically small dimeric enzymes with a preponderance of positively charged residues. At least for some resolvases these charges form a largely basic electrostatic surface, which presumably enables interaction with the overall shape of the DNA junction. Enzymatically the resolvases are characterized by their ability to: 1) bind Holliday junctions in a structure-specific manner; 2) introduce a pair of symmetrically related nicks in strands of like polarity at or close to the junction crossover point; and 3) generate nicks with 5′-phosphate and 3′-hydroxyl termini that are readily ligated. In most cases junction binding is associated with a distortion of the stacked-X conformation of the Holliday junction to a more open structure (8–13). This is thought to aid the positioning of the scissile bonds close to the catalytic sites of the enzyme. A subset of resolvases, including Escherichia coli RuvC, which are most selective for cleaving Holliday junctions, only cut at specific nucleotide sequences. In the case of RuvC this sequence is 5′-AT/TT (14, 15). Sequence specificity is proposed to aid structure selectivity because, unlike many other branched DNA molecules, Holliday junctions can be moved to different nucleotide sequences by branch migration. In accord with a need for relocating junctions to cleavable sequences, RuvC functions together with RuvAB that catalyzes the ATP-dependent branch migration of Holliday junctions (16, 17).

The Saccharomyces cerevisiae cruciform-cutting endonuclease 1 (CCE1) and its homologue from Schizosaccharomyces pombe SpCCE1 (also called Ydc2) are mitochondrial-specific Holliday junction resolvases (18–22). Despite displaying little sequence identity, CCE1 and SpCCE1 are structurally related to RuvC (23, 24). Like RuvC they unfold the Holliday junction into a similar square-planar extended structure and cleave it with clear sequence specificity (after 5′-CT and/or 5′-TT in the case of SpCCE1) (8, 9, 21, 25, 26). However, unlike RuvC, these enzymes appear to function efficiently in vivo without the need for a specific branch migration enzyme.

In this paper we have investigated the role of a putative DNA binding domain in SpCCE1 that resembles the SAP motif (named after SAF-A/B, Acinus and PIAS) (27). The SAP motif is a helix-extended region helix domain that contains a number of conserved hydrophobic and charged amino acids as well as one or two conserved glycines in the extended loop region, and is typically located at the N or C terminus of a protein. SAP motifs are found in a number of chromatin-associated proteins, such as the scaffold attachment factors SAF-A and SAF-B, PIAS family members, Acinus, and the proto-oncogene protein DEK (27). Typically these proteins function as transcription factors and/or are involved in RNA metabolism. The SAP motif is also found in several DNA repair proteins, including the non-homologous end-joining protein Ku70, some plant homologues of poly(ADP-ribose) polymerase, the AP endonuclease Arp, and the post-replication repair protein Rad18 (27).

Direct evidence that the SAP motif is important for DNA binding has been obtained for SAF-A and Ku70. In both cases DNA binding, which is dependent on SAP, could be detected by Southwestern blotting and pull-down assays using protein coupled to Sepharose (28–31). The SAP motif of SAF-A binds specifically to DNA elements called scaffold/matrix attachment regions, which are chromatin regions that bind to the nuclear matrix (30). On the basis of this it has been suggested that the SAP motif may generally target proteins involved in transcrip-
tion, pre-mRNA processing, and DNA repair to specific regions of the chromatin (27, 30).

Here we show that the SAP motif is critical for the ability of SpCCE1 to resolve Holliday junctions in a heterologous in vitro system. The dependence of SpCCE1 on SAP is not for targeting it to scaffold/matrix attachment regions or Holliday junctions, or even resolving junctions per se. Instead the SAP motif is required for stabilizing the interaction of SpCCE1 with a Holliday junction. We provide indirect evidence that stable junction binding is necessary to promote branch migration to cleavable sites. As such SpCCE1 may be the first example of a Holliday junction resolvase that couples branch migration and cleavage activities in a single polypeptide.

EXPERIMENTAL PROCEDURES

Isolates, Strains, and General Methods—The E. coli strains AM888 (∆ruvAC65 ∆sak::kan) and BL21(DE3) pLYS8 have been described, as has the plasmid vector pTT-7 and its SpCCE1-derivative pMW206, and pMW207, which is a derivative of pET-14b (Novagen) that expresses SpCCE1 with an N-terminal His tag from a T7 phage promoter (20, 45, 48). Strains were grown in LB medium with antibiotic selection as required. Measurement of UV sensitivity was as described (20).

Construction of SpCCE1 SAP Motif Mutants—The N-terminal deletion mutants were constructed by PCR using primers that amplified truncated versions of SpCCE1 which lack the first 5 (Δ5), 15 (Δ15), and 32 (Δ32) codons. In each case an ATG start codon was incorporated to allow expression of the truncated protein, along with NdeI and BamHI sites to facilitate cloning into pTT-7 and pET-14b as described previously for pMW206 and pMW207. Single and double amino acid substitutions in SpCCE1 were made by introducing appropriate codon changes in the SpCCE1 gene in each of the plasmids generated. Derivatives of pTT-7 containing the mutant SpCCE1 genes were made by subcloning from the mutated pMW207 plasmids.

Proteins—His-tagged wild-type and mutant SpCCE1 proteins were expressed from the appropriate pET-14b-derived SpCCE1 plasmid in BL21 (DE3) pLYS8. Cells were grown as 1-l culture at 18 °C in LB medium containing 100 μg/ml ampicillin and 20 μg/ml chloramphenicol. At a cell density corresponding to an A600 of 0.5, SpCCE1 was induced by adding isopropyl-1-thio-β-D-galactopyranoside to 1 mM. Incubation was continued for a further 80 min, after which the harvested, resuspended in 20 ml of buffer (50 mM potassium phosphate, pH 8.0, 300 mM NaCl, 10% glycerol) and frozen at −80 °C until required. Frozen cells were thawed on ice, mixed with β-mercaptoethanol (to 7 mM), Triton X-100 (to 1%), and protease inhibitors, before lysis by passage through a French pressure cell at 15,000 p.s.i. After centrifugation at 43,700 × g for 1 h the clarified lysate was loaded directly onto a 1-mL nickel-nitrioltriacetic acid Superflow column (Qiagen), which was washed with 30 ml of buffer H (50 mM potassium phosphate, pH 8.0, 7 mM β-mercaptoethanol, 300 mM NaCl, 10% glycerol) containing 50 mM imidazole before eluting bound protein with 4 ml of buffer H containing 200 mM imidazole. The nickel eluate was loaded onto an 1-mL HiTrap SP-Sepharose column (Amersham Biosciences) equilibrated with buffer A (50 mM Tris- HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) containing 0.1 M NaCl. After washing the column with 5 ml of the equilibration buffer bound proteins were eluted with a linear gradient of 0.1 to 1.0 M NaCl in buffer A. Wild-type and mutant SpCCE1 proteins eluted within the range of 500 to 850 mM NaCl. In each case the proteins were pooled and dialysed against protein storage buffer (50 mM Tris- HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 0.1 M NaCl, 50% glycerol) before aliquoting and storing at −80 °C. Protein samples were judged to be ≥95% homogeneous by standard SDS-polyacrylamide gel electrophoresis analysis with Coomassie Blue staining. Amounts of protein were estimated using a modified Bradford assay from bovine serum albumin as the standard, and are expressed in molar concentrations of monomer.

DNA Substrates—Synthetic Holliday junctions X-12 and X-26, with 12- and 26-bp mobile cores, respectively, were made from 50-mer oligonucleotides as described (32, 49, 50). In the case of X-12 oligonucleotide 2 was labeled with 32P at its 5′ end. Four different preparations of X-26 were made each labeled on a different oligonucleotide so that all cleavage sites could be mapped (see below). The linear duplex was made from oligonucleotides 2 and 13 (49). The junction (J1) used for the analysis of the SpCCE1-junction complex formation has been described, as has the preparation of the six junction species that are derived by its restriction digestion (34). χ DNA, which contains a Holliday junction within a ~300-bp homologous core flanked by heterologous arms of 0.8–1.6 kb, was made as described (4). This substrate is labeled with 32P on each of its four arms.

X-12 and X-26 Cleavage Assays—Reactions for monitoring the rate of X-12 cleavage contained 0.44 nm X-12 in binding buffer (25 mM Tris- HCl, pH 8.0, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 6% glycerol), plus 200 μM NaCl, 10 mM MgCl2, and 5 nm SpCCE1 in a total volume of 70 μl. After precipitation of the reaction mixture at 37 °C for 5 min the reaction was started by the addition of SpCCE1. 10-μl samples were withdrawn at the indicated times and stopped by the addition of one-fifth volume of stop mixture (2.5% SDS, 200 mM EDTA, 10 mg/ml proteinase K) followed by a further 10 min at 37 °C. DNA products were analysed by electrophoresis through a 10% polyacrylamide gel. Reactions for comparing the cleavage sites made by wild-type and R23G proteins on X-26 were essentially the same as described above except that reaction volumes were 40 μl, contained 10 nm protein and were incubated for 15 min before being stopped. For each protein four reactions were run in parallel each using a different preparation of DNA. DNA products were processed and then analyzed on a 15% denaturing gel as described. All gels were dried and analyzed by phosphoimagining on a Fuji FLA3000.

DNA Binding Assays—Reaction mixtures (20 μl) contained 0.44 nm labeled X-12 or linear duplex in binding buffer plus 200 mM NaCl. After adding SpCCE1 as indicated, reactions were left on ice for 10 min before loading on a gel in low ionic strength buffer (6.7 mM Tris- HCl, pH 8.0, 3.3 mM sodium acetate, 2 mM EDTA). Gels were run at 160 V for 105 min, dried, and analyzed as described. For dissociation assays reaction mixtures (60 μl) contained X-12 in binding buffer plus 200 mM NaCl and 15 nm SpCCE1 as above. Reactions were incubated at 22 °C for 5 min prior to loading 10 μl on a 4% gel running at 200 V. 16Gels were dried and analyzed as described. Molecular modeling of the SpCCE1 dimer was made using the program RasMol (24). Molecular modeling of the SpCCE1 crystal structure onto a Holliday junction, in its approximately square planar “unstacked” conformation, reveals the potential of this helical domain contacting a junction arm via its positively charged surface (24). The presence of helical domains on either side of the SpCCE1 dimer would ensure that...
two opposing junction arms are contacted (Fig. 1B). We obtained further support for this domain playing a role in junction binding from a RPS-BLAST search of the Conserved Domain Data base (NCBI). This revealed that the N-terminal helices 1 and 2, together with their connecting loop, matches the consensus for the putative DNA binding motif SAP (Fig. 1C) (see Introduction). To further investigate the potential role of the SAP motif in junction binding by SpCCE1, a series of mutant proteins was constructed that contained either deletions or specific amino acid substitutions in this N-terminal region (Fig. 1D). These mutant proteins were then tested in vitro and in vivo as described below.

The SAP Motif Is Important for the Ability of SpCCE1 to Resolve Holiday Junctions in Vivo—SpCCE1 is encoded in the nucleus but appears to function exclusively in the mitochondria of fission yeast (22). Deletion of SpccE1 does not result in any overt phenotype, except for an increase in the aggregation of mitochondrial DNA molecules, which is detectable by cytological and Southern blot analysis (22). To provide a more sensitive in vivo assay for comparison of the SpCCE1 mutant proteins, we made use of the ability of SpCCE1 to complement the hypersensitivity to UV light of a resolvase-deficient E. coli strain (20). A ruvAC− ruvA− strain (AM888), which is deleted for both known Holliday junction resolvases in E. coli, was transformed with the plasmid pT7-7 and derivatives expressing SpCCE1 proteins. The effect of these plasmids on the survival of AM888 following UV irradiation was then measured (Fig. 2 and Table I). As expected AM888 carrying pT7-7 is very sensitive to UV light, whereas the same strain carrying pMW206, which expresses wild-type SpCCE1, is relatively resistant (Fig. 2A). In fact SpCCE1 is such a good substitute for RuvC and RusA that it improves

Fig. 1. Identification of a SAP motif at the N terminus of SpCCE1. A, structure of the SpCCE1 dimer generated from the coordinates in the Protein Data Bank (accession code 1KCF) using VMD software. Helices are represented as red cylinders. Helix 1 and 2 are labeled, as are the N and C termini. B, model of the SpCCE1-Holliday junction complex as proposed by Ceschini et al. (24). The model shows how the SpCCE1 dimer (colored in orange and purple) is thought to dock on one face of the Holliday junction in an open, approximately square-planar conformation. It also shows how the SAP domains (colored in purple) contact opposing junction arms. C, alignment of SAP motifs from different proteins constructed using ClustalW. Solid blocks indicate identical residues found in >60% of the proteins shown, whereas gray blocks indicate similar residues. The proteins used for the alignment represent only a selection of those containing a SAP motif in the Conserved Domain Data base (NCBI). Most of these are mentioned in the Introduction apart from E1B 55 kDa and THO1 (27). Protein names, shown on the left, are prefixed by a two-letter abbreviation of the species that they are from. These are S. pombe (Sp), Mus musculus (Mm), Homo sapiens (Hs), S. cerevisiae (Sc), and Arabidopsis thaliana (At). D, SpCCE1 SAP motif mutants. The various amino acid changes and deletions are shown in relation to the primary and secondary structure of the SAP motif in SpCCE1. The three N-terminal deletions are shown in red.

Fig. 2. Effect of SAP motif mutants on the UV sensitivity of an E. coli ruv− ruvA− strain. A and B, survival curves of AM888 (ΔruvAC65 ΔruvA::kan) carrying the vector pT7-7 and derivatives encoding wild-type and mutant SpCCE1 proteins as indicated. Values are the means of at least two independent experiments. Data for only three mutant SpCCE1 proteins is shown but each of these is representative of a particular subset of mutants as summarized in Table I.
Role of the SAP Motif in SpCCE1

In vivo and in vitro activities of wild-type and mutant SpCCE1 proteins

| SpCCE1       | Suppression of ruw sensitivityb | UV Relative rate of cleavage of X-12c | Binding affinity for X-12f | Off-rate of SpCCE1-X-12 complexesb | Unfolds stacked X-junctions (see Fig. 6) |
|--------------|---------------------------------|-------------------------------------|---------------------------|-----------------------------------|----------------------------------------|
| Wild-type    | +                               | 1.0                                 | 3.5                       | 0.8                               | Yes                                    |
| Δ5           |                                 | 0.43                                | 2                         | -1.7                              | ND                                     |
| Δ15          |                                 | 0.08                                | 5.5                       | <0.25                             | Yes                                    |
| Δ32          |                                 | ND                                  | ND                        | ND                                | ND                                     |
| V4P          | +                               | 0.89                                | 3                         | >10                               | ND                                     |
| L9P          | +                               | 0.97                                | 1.5                       | >10                               | ND                                     |
| K14A         | +                               | 0.8                                 | 1.5                       | >10                               | ND                                     |
| L9P/K14A     |                                 | 0.007                               | 6                         | <0.25                             | Yes                                    |
| L18G         |                                 | 0.18                                | 2                         | <0.25                             | ND                                     |
| G22S         |                                 | 0.36                                | 5                         | <0.25                             | Yes                                    |
| G22D         |                                 | ND                                  | ND                        | ND                                | ND                                     |
| R23G         |                                 | 1.57                                | 2.5                       | <0.25                             | Yes                                    |
| K24G         |                                 | 0.69                                | 4.5                       | <0.25                             | ND                                     |
| R23G/K24G    |                                 | 0.07                                | 6.8                       | <0.25                             | ND                                     |
| L27P         |                                 | ND                                  | ND                        | ND                                | ND                                     |

a An E. coli ruw: ruA- strain (AM888) carrying the pT7-7 vector or derivatives encoding wild-type or mutant SpCCE1 proteins were assessed for their UV sensitivity as described under "Experimental Procedures." + indicates suppression of UV sensitivity; ± indicates partial suppression; and – indicates no suppression (see Fig. 2).
b Rates of cleavage of X-12 are relative to a nominal value for wild-type SpCCE1 of 1.0, and were calculated from the data in Fig. 3.
c Apparent dissociation constants (Kd) for SpCCE1-X-12 complexes were estimated from data such as in Fig. 4.
d The off-rate of SpCCE1-X-12 complexes is the time taken for 50% of the protein-junction complexes to dissociate, and was calculated from data such as shown in Fig. 5.
e The ability to unfold junction J1 into an open square planar conformation in the presence of 200 μM MgCl2 (see Fig. 6).
f ND, not determined.

Survival of AM888 by almost 1000-fold following irradiation with 40 J/m² of UV (Fig. 2A).

Deletion of part or most of the SAP motif (proteins Δ15 and Δ32, respectively) destroys the ability of SpCCE1 to suppress the UV sensitivity of AM888 (Table I). SpCCE1 is similarly affected by single amino acid changes in four of six conserved residues that were analyzed (L18G, G22S, G22D, K24G, and L27P), and one non-conserved residue (R23G) (Fig. 2B and Table I). Furthermore, deletion of the first five amino acids (Δ5) significantly impairs SpCCE1 in vivo (Fig. 2B). In contrast, the mutations V4P, L9P, and K14A have no effect on the ability of SpCCE1 to suppress the UV sensitivity of AM888 (Fig. 2B and Table I). Although, combining L9P and K14A mutations does render SpCCE1 inactive in this assay (Table I). Taken together these data indicate that the SAP motif in SpCCE1 is required for efficient resolution of Holliday junctions in an in vivo system.

Impaired Cleavage of Holliday Junctions by SpCCE1 SAP Motif Mutants—To investigate how mutations in the SAP motif affect SpCCE1, His-tagged wild-type and mutant proteins were purified. Three of the mutant proteins (Δ32, G22D, and L27P) proved to be insoluble so were not studied further. The 11 remaining mutant proteins were tested for their ability to resolve the small synthetic Holliday junction substrate X-12.

Wild-type SpCCE1 cleaves X-12 extremely well generating increasing amounts of nicked linear duplex product over the 5-min time course of the reaction (Fig. 3, A, lanes a–f, and B). V4P, L9P, and K14A mutant proteins, which fully suppress the UV sensitivity of AM888, also cleave X-12 efficiently (Fig. 3, B and C). Seven mutant proteins (Δ5, L9P/K14A, Δ15, L18G, G22S, K24G, and R23G/K24G) show various reduced cleavage rates (Fig. 3, A, lanes g–l, C–E). Each of these is partially or completely defective for suppression of AM888 UV sensitivity (Table I). However, there is an imperfect correlation between the cleavage rate of X-12 and in vitro activity; e.g., Δ5, G22S, and K24G each have similar cleavage rates in vitro yet only Δ5 retains any ability to suppress AM888 UV sensitivity (Table I). Furthermore, R23G, which cleaves X-12 at least as well as the wild-type protein (Fig. 3E), is apparently impotent in vivo (Fig. 2B).

Binding of Holliday Junctions by SpCCE1 SAP Motif Mutants—Because the SAP motif is predicted to be generally required for DNA binding, it was likely that the reduced in vivo and in vitro activity of the SpCCE1 mutant proteins was because of impaired binding to Holliday junctions. To investigate this we measured the ability of the mutant proteins to bind X-12 using a standard band-shift assay. Wild-type SpCCE1 binds to X-12 to form two complexes (Fig. 4A, lanes a–g). Complex 1 is formed by the binding of a single dimer of SpCCE1 to X-12, whereas, complex 2 is formed by the binding of two dimers (20, 21, 32). For each mutant protein we observed complexes with the same mobility as complexes 1 and 2 formed by wild-type SpCCE1 (Fig. 4A, lanes h–u, and data not shown). These data indicate that the mutant proteins retain the ability to dimerize and bind to Holliday junctions. The affinity of the mutants for X-12 also remains similar to wild-type (Fig. 4B and Table I). This is not only true of V4P, L9P, and K14A, which appear to cleave Holliday junctions in vitro and in vivo just as well as wild-type, but also the mutants that have impaired functionality. In particular, Δ5, L18G, and R23G exhibit the same binding affinity as wild-type SpCCE1 (Table I). Only Δ15, L9P/K14A, G22S, K24G, and R23G/K24G bind slightly less well than wild-type. These data are not consistent with the idea that the SAP motif plays a critical role in junction binding by SpCCE1.

The SAP Motif Is Required for Stabilizing the Interaction of SpCCE1 with Holliday Junctions—Band-shift gels can help to stabilize certain protein-DNA interactions by creating a "caging" effect, where the local concentration of protein and DNA in the gel is such that re-association of dissociated complexes is strongly favored (33). For this reason it is possible that the standard band-shift analysis is not sensitive enough to detect differences in junction binding by the mutant proteins. We therefore used an "off-rate" assay in which the dissociation of SpCCE1-X-12 complexes was measured following the addition of an excess of unlabeled double-stranded (ds)DNA. In this assay the dsDNA acts as a passive sink for protein that has dissociated from X-12, and is added in sufficient excess to limit junction binding to ~10% if added before the protein (Fig. 5A, 1).
lanes g, h, o, and p, and data not shown). For all proteins complex 2 rapidly dissociates following addition of the dsDNA (Fig. 5A and data not shown). However, wild-type SpCCE1X-12 complex 1 remains relatively stable with a half-life of 8 min (Fig. 5, A, lanes a–f, B and Table I). Similar half-lives are observed for the wild-type-like mutants V4P, L9P, and K14A (Table I). In contrast, the mutants, which show little or no suppression of AM888 UV sensitivity, form unstable complexes with X-12 (Table I). Two classes of mutant can be distinguished. The first is characterized by its sole member Δ5, which has a half-life on X-12 of ~1.7 min (Table I), whereas the second is exemplified by R23G, which has a half-life on X-12 of less than 15 s (Fig. 5, A, lanes i–n, and B). However, it is worth emphasizing that other SAP motif mutants may form even less stable complexes with X-12 than R23G, because this gel assay can only determine half-lives ≥15 s. The relative stability of the junction complexes formed by these two classes of mutant protein correlates well with their ability to suppress AM888 UV sensitivity, i.e. Δ5 partially suppresses, whereas the mutants typified by R23G provide no suppression (Fig. 2B and Table I).

**Holliday Junction Specificity Is Unaltered by Mutation of the SAP Motif**—The reduced stability of the mutant protein-junction complexes seen in Fig. 5 could be explained by a relative increase in binding affinity for duplex DNA. However, a comparison of wild-type and mutant proteins for binding to a linear dsDNA revealed no differences (data not shown). Furthermore, similar amounts of dsDNA are required for competing off both wild-type and mutant proteins from X-12 (data not shown). These data indicate that the SAP motif mutants retain a wild-type level of binding specificity for Holliday junctions.

**SAP Motif Mutants Unfold Holliday Junctions Like Wild-type SpCCE1**—In the absence of divalent cations charge repulsion between the arms of a Holliday junction forces it into an approximately planar 4-fold symmetrical arrangement in which each arm subtends an angle of about 90° with its neighbor (34). With as little as 100 μM Mg²⁺ the repelling charges are neutralized and the junction is able to adopt the so-called stacked-X structure, in which junction arms stack pairwise and form a right-handed cross with continuous and exchanging strands in an antiparallel arrangement (34, 35). These alternate conformations of the junction can be detected by a method of comparative gel electrophoresis (34). This uses six junction species derived from different restriction digests of the same X-junction (Fig. 6A). Each junction species contains a different
pair of long (40 bp) and short (15 bp) junction arms, with the angle subtended between the two long arms being the major factor in determining its relative gel electrophoretic mobility. In the absence of Mg\textsuperscript{2+}, the six junction species migrate with a four-slow, two-fast pattern characteristic of the open square configuration of the junction (Fig. 6B, lanes a–f). In the presence of Mg\textsuperscript{2+} this pattern changes to two-slow, two-intermediate, and two-fast, which is characteristic of the stacked-X structure (Fig. 6C, lanes a–f).

Holliday junctions bound by wild-type SpCCE1 adopt an open square planar configuration irrespective of the presence of metal ions (9). The prediction that two opposing junction arms would be contacted by SAP motifs on either side of the SpCCE1 dimer, with the potential for forming interactions between arginines/lysines (e.g. Arg-23, Lys-24, etc.) and phosphate groups (24), suggests that the SAP motif could be required for SpCCE1 to properly unfold the stacked X-junction. However, comparative gel analysis of the mutant proteins, bound to the six different junction species, reveals the same pattern of four-slow and two-fast complexes that is seen with wild-type SpCCE1 (Table I). These data show that the SAP motif is not required for SpCCE1 to unfold stacked Holliday junctions.

Stable Holliday Junction Binding Is Required for Efficient Cleavage of \(\chi\) DNA—So far the only in vitro defect in the SAP motif mutants that correlates in all cases with their ability to function in vivo is the instability of their interaction with X-12. This is exemplified by R23G, which is unable to suppress the UV sensitivity of AM888 and forms an unstable complex with X-12, yet cleaves X-12 as well as wild-type SpCCE1. At least for R23G the stability of junction binding is not a prerequisite for efficient Holliday junction cleavage in vitro, so why might it be important in vivo?

One possibility is based on the idea that SpCCE1 might promote the branch migration of Holliday junctions to cleavable sites by holding them in an open configuration (24). This would be less critical for cleaving X-12, which contains several SpCCE1 cleavage sites within its 12-bp homologous core, but could be necessary for processing junctions that are more remote from cleavable sites. To test this idea we compared the rate of cleavage of \(\chi\) DNA by wild-type and R23G proteins. \(\chi\) contains a Holliday junction within a 300-bp homologous core with 43 preferred cleavage sites (5'-CT and 5'-TT dinucleotides) for SpCCE1. These sites are unevenly distributed throughout the core, which leaves 9 regions, between 12 and 29 bp long,
that do not contain a preferred cleavage site (data not shown).

Cleavage reactions were performed at 25 °C in the presence of 10 mM MgCl₂ to limit spontaneous branch migration. Under these conditions the R23G mutant cleaves χ at approximately a quarter of the rate of wild-type SpCCE1 (Fig. 7, A and C). In contrast, R23G cleaves X-12 slightly faster than wild-type under these conditions (Fig. 7, A and C). These data are consistent with R23G being less able to promote branch migration of Holliday junctions to cleavable sites. Alternatively, R23G may have a more stringent sequence preference for cleavage, and therefore be unable to cleave a subset of sites that are cleaved well by wild-type SpCCE1. To rule out this possibility, we mapped the cleavage sites of wild-type and R23G proteins on a synthetic Holliday junction with a 26-bp homologous core (X-26). Both enzymes cleave X-26 with exactly the same site preferences (data not shown).

If the difference between wild-type and R23G proteins for cleaving χ is because of a deficiency in R23G promoting branch migration, then conditions, such as increased temperature, which promote spontaneous branch migration, should reduce the imbalance. This is exactly what is observed. At 42 °C R23G cleaves χ almost as well as wild-type SpCCE1 (Fig. 7, B and C), whereas cleavage of X-12 remains faster than wild-type (Fig. 7, B and C).

**DISCUSSION**

In this paper we have shown that SpCCE1 contains a putative DNA binding domain, called a SAP motif, at its N terminus. Mutation or deletion of this motif prevents or limits the ability of SpCCE1 to resolve Holliday junctions in vivo. These mutant proteins are variably affected for junction binding and resolution in vitro. However, their primary defect appears to be a reduction in the stability of junction binding. The analysis of one mutant protein (R23G), in particular, shows that binding stability is not a prerequisite for efficient junction cleavage. Instead, stable junction binding may be required for promoting the spontaneous branch migration of Holliday junctions to cleavable sites.

**The Role of the SAP Motif in Stabilizing Protein-DNA Interactions**—The SAP motif has recently been recognized as a new type of eukaryotic DNA binding domain. It is found in proteins involved in nuclear architecture, transcription, RNA processing, and DNA repair (27). Direct evidence that the SAP motif binds DNA has come from studies of SAF-A and Ku70 (28–31). In both cases deletion of the SAP motif results in loss of DNA binding detected by Southwestern blotting and immunoprecipitation. Furthermore, the SAP motif from SAF-A has been synthesized as a peptide and shown to bind specifically to the scaffold/matrix attachment regions by immunoprecipitation, thus demonstrating that SAP is an independent DNA binding domain (30).

SpCCE1 is only the third protein in which the role of the SAP motif has been investigated, and the first in which single amino acid changes have been used to dissect structure-function relationships. As with SAF-A and Ku70, deletions in the SAP motif affect the interaction of SpCCE1 with DNA. In the case of SpCCE1, the SAP motif is not the main DNA binding determinant, instead it is used to stabilize the active SpCCE1-junction complex. Such a role is consistent with molecular modeling of the SpCCE1 crystal structure on a Holliday junction in an open, square planar conformation, which shows how positively charged residues in the SAP motif could make ionic and hydrogen bonding interactions with the sugar-phosphate backbone of a junction arm (24). The position of the two SAP motifs in the SpCCE1 dimer would ensure that similar interactions were made in two opposing junction arms (Fig. 1B). Basic residues that are predicted to play key roles include Arg-23 and Lys-24 (24). Indeed replacement of either residue with glycine dramatically reduces the half-life of the SpCCE1-junction complex (Table I), with the K24G mutant apparently more impaired than R23G based on its lower rate of X-12 cleavage (Fig. 3E).

The combination of R23G and K24G mutations reduces X-12 cleavage still further indicating that each residue contributes independently to binding stability. The fact that Lys-24 appears to be more important is consistent with it being one of the
most conserved residues in the SAP motif. In contrast a basic residue in the Arg-23 position is found in only 12 of 43 SAP motif proteins in the NCBI Conserved Domain Data base (data not shown).

It is now clear from the analysis of three different proteins that the SAP motif can be used either as the main DNA binding component of a protein (e.g. SAP-A) or as an additional stabilizing element (e.g. Ku70 and SpCCE1). A single SAP domain forms a relatively weak interaction with DNA, which is undetectable in solution (30). However, when many SAP domains are brought into close proximity (e.g. by immobilization on a membrane or Sepharose beads) cooperative effects lead to high affinity DNA binding (30). So proteins that use a SAP domain as their main or sole DNA binding element must form large multimers to achieve the necessary cooperative effects for binding. This appears to be the case for SAP-A that self-assembles into long filamentous or globular complexes (36, 37). In contrast, Ku70 functions as a heterodimer with Ku80 forming a ring structure that binds to DNA ends to promote non-homologous end-joining (38, 39). Here, as with SpCCE1, the SAP domain is not the principle DNA binding determinant, instead it may provide a barrier to the inward movement of Ku from a DNA end, or to cause pausing of Ku at specific sequences (39).

Branch Migration and Resolution by SpCCE1—All junction resolvases recognize the structure of the Holliday junction. For some, like T4 endonuclease VII and T7 endonuclease I, simply binding to this structure is sufficient to trigger cleavage (7). However, a subset of resolvases, including RuvC, RusA, CCE1, and SpCCE1, require specific nucleotides to be present at the junction crossover point before they will cleave (12, 14, 15, 20, 21, 25, 26, 40). Consequently, these resolvases depend on the ability of the Holliday junction to branch migrate so that the required sequence can be localized at the junction point. Branch migration involves the breakage and reformation of an equal number of hydrogen bonds between base pairs. As such it is an isoenenergetic process and can occur spontaneously. However, in the presence of physiological levels of Mg2+, the junction folds into the stacked-X structure, which slows branch migration by ~1000-fold (41–43). Therefore efficient junction resolution by the “sequence-specific” resolvases must depend on some mechanism for promoting branch migration. This dependence is seemingly made more acute for RusA and SpCCE1, which form long-lived protein-junction complexes that could inhibit resolution if the junction is not already localized at a sequence that is cleavable (32, 44).

The solution of RuvC for ensuring that Holliday junctions can be moved to sites at which it can cleave, is to associate with a dedicated branch migration enzyme RuvAB (16, 17). RusA, which can substitute for RuvABC in E. coli, does not associate with a specific branch migration enzyme in the same way that RuvC does, but does depend partly on RecG, which can branch migrate Holliday junctions (45, 46). It is not known whether CCE1 or SpCCE1 function together with a specific branch migration enzyme in the mitochondria of yeast. However, if such an association does exist, then at least for SpCCE1, it is not a prerequisite for resolving Holliday junctions in vivo, because this enzyme can efficiently substitute for RuvABC and RusA in E. coli (Fig. 2).

In the absence of a branch migration enzyme SpCCE1 may have evolved another means of moving Holliday junctions around. Evidence for this has come from our study of the SAP motif mutants. These mutants are able to recognize, bind, and unfold the Holliday junction-like wild-type protein but unlike wild-type SpCCE1, which forms a relatively long-lived complex with junction DNA, the mutant proteins rapidly dissociate after binding. For the majority of the mutants tested, binding stability appears to be reduced sufficiently to affect their rate of X-12 cleavage. Presumably the mutant proteins have a greater tendency to dissociate from the junction before they have had a chance to cleave it. Analysis of the R23G mutant indicates an additional importance for stable junction binding. Unlike the other SAP motif mutants that are defective for resolving Holliday junctions in vivo, R23G cleaves X-12 at least as well as wild-type protein. This demonstrates that binding stability can be reduced more than 30-fold without affecting the efficiency of junction resolution. However, R23G cleaves γ DNA at a much slower rate than wild-type protein. In γ the junction point can be located more distantly from a cleavable site than in X-12, therefore a reduced ability to cleave γ suggests that R23G might have a problem relocating junctions to cleavable sites. This interpretation is supported by the fact that conditions that promote spontaneous branch migration improve the ability of R23G to cleave γ. Based on these data we speculate that Holliday junctions remain mobile when bound to SpCCE1. Furthermore, this mobility may be enhanced by the junction being held in an open configuration, which favors branch migration. Such branch migration, being a passive process, would lack directionality, and be impeded by base-base mismatches and other protein complexes. Nevertheless, it should be sufficient to locate the junction to one of the preferred cleavage sites of SpCCE1, which on average would be within 8 bp of the junction crossover point.

SpCCE1 is the only known Holliday junction resolvase that contains a SAP motif. This suggests that the SAP motif may have been acquired latterly during the evolution of SpCCE1. Possibly SAP is ideal for stabilizing protein-DNA interactions while allowing a process such as spontaneous branch migration. However, the fact that the homologue of SpCCE1 in budding yeast CCE1 does not contain a SAP motif (20), suggests that there are other means of promoting stable junction binding while potentially retaining junction mobility.

We have speculated above that SpCCE1 promotes spontaneous branch migration by holding the junction in an open conformation. Both RuvC and RusA, like (Sp)CCE1, unfold the Holliday junction upon binding (11, 12, 44). It is therefore possible that they too could promote spontaneous branch migration. However, (Sp)CCE1 appears to open out the junction much more than the other resolvases, holding it in an –4-fold symmetric structure reminiscent of the RuvA-junction complex (8, 9). Furthermore, CCE1 (and probably SpCCE1), unlike RuvC and RusA, breaks all 4 base pairs that surround the point of strand exchange (47). These additional conformational changes may mean that (Sp)CCE1 is more adept at promoting spontaneous branch migration than either RuvC or RusA.

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