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Peptide Inhibitors of DNA Cleavage by Tyrosine Recombinases and Topoisomerases

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The study of biochemical pathways requires the isolation and characterization of each and every intermediate in the pathway. For the site-specific recombination reactions catalyzed by the bacteriophage λ tyrosine recombinase integrase (Int), this has been difficult because of the high level of efficiency of the reaction, the highly reversible nature of certain reaction steps, and the lack of requirements for high-energy cofactors or metals. By screening synthetic peptide combinatorial libraries, we have identified two related hexapeptides, KWWCRW and KWWWRW, that block the strand-cleavage activity of Int but not the assembly of higher-order intermediates. Although the peptides bind DNA, their inhibitory activity appears to be more specifically targeted to the Int-substrate complex, insofar as inhibition is resistant to high levels of non-specific competitor DNA and the peptides have higher levels of affinity for the Int-DNA substrate complex than for DNA alone. The peptides inhibit the four pathways of Int-mediated recombination with different potencies, suggesting that the interactions of the Int enzyme with its DNA substrates differs among pathways. The KWWCRW and KWWWRW peptides also inhibit vaccinia virus topoisomerase, a type IB enzyme, which is mechanistically and structurally related to Int. The peptides differentially affect the forward and reverse DNA transesterification steps of the vaccinia topoisomerase. They block formation of the covalent vaccinia topoisomerase-DNA intermediate, but have no apparent effect on DNA religation by preformed covalent complexes. The peptides also inhibit *Escherichia coli* topoisomerase I, a type IA enzyme. Finally, the peptides inhibit the bacteriophage T4 type II topoisomerase and several restriction enzymes with 2000-fold lower potency than they inhibit integrase in the bent-L pathway.

Keywords: inhibitory peptides; bacteriophage λ integrase; vaccinia topoisomerase; *E. coli* topoisomerase I; type II topoisomerase

Introduction

Site-specific recombination reactions are widespread in nature and are used to control gene expression, amplify episome copy number, create genetic diversity, and separate chromosomes at bacterial cell division (reviewed by Landy, 1989; Nash, 1996). Some of these reactions, exemplified by recombination of bacteriophage P1 *lox* sites by the phage-encoded Cre recombinase, appear stochastic and are bi-directional (the products are the same as the substrates). Other reactions, exemplified by bacteriophage λ site-specific recombination, use more complex substrates to integrate and excise prophages into and out of the host chromosome in a manner responsive to host cell physiology. These integrative and excisive recombination reactions are unidirectional (the products differ from the substrates) and involve accessory factors encoded by the phage (e.g. excisionase (Xis)) and by the host (e.g. the integration host factor (IHF) (see Figure 1 and Table 1). The bacterio-

Abbreviations used: Int, integrase; IHF, integration host factor; Xis, excisionase; FIS, factor for inversion stimulation; UMC, unimolecular complex; BMC, bimolecular complex; CPD, covalent protein-DNA complex; HJ, Holliday junctions.

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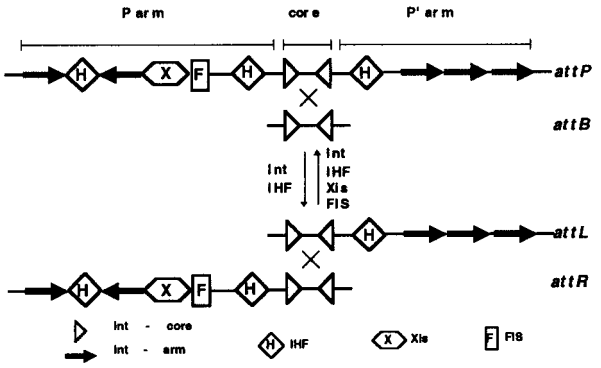

Figure 1. DNA substrates and proteins necessary for bacteriophage λ integrative and excisive recombination.

phage λ site-specific recombinase, integrase (Int), also catalyzes two bi-directional recombination reactions known as straight-L and bent-L, in which the substrates are identical to the products (Table 1). Int's ability to act in the context of complexes between different pairs of recombination substrates is still incompletely understood at the molecular level.

Phage λ integrase (Int) belongs to a large family of tyrosine recombinases (Esposito & Scocca, 1998; Nunes-Duby *et al.*, 1998; Burgin & Nash, 1995; Kitts & Nash, 1988). These enzymes mediate catalysis by two rounds of single-strand cleavage, exchange, and ligation (reviewed by Nash, 1996; see Figure 1 of the accompanying paper by Cassell *et al.*, 2000). A Holliday junction intermediate between the two DNA substrates is generated after the first round of catalysis and is resolved by the second the degree of homology is used at the strand exchange step as a way to test the suitability of substrate DNA molecules: if the substrates are not identical in the seven-base-pair overlap region between the loci of strand cleavage and ligation, the reaction is quickly reversed to starting substrates (Kitts & Nash, 1987; Nunes-Duby *et al.*, 1995). This reversibility, plus the fact that these reactions require no external high-energy cofactors for binding or catalysis, have made it difficult to understand which reaction steps are rate limiting or to isolate and analyze reaction intermediates.

Several approaches have been used to block the reaction at early stages, including modifications of DNA substrates (Kitts & Nash, 1987; Nash *et al.*, 1987; Pargellis *et al.*, 1988) or using cleavage-defective Int mutants, for example IntF (Y342F) (Pargellis *et al.*, 1988; Han *et al.*, 1994). Each of these strategies has limitations. The DNA modifications can only be easily introduced into linear substrates, and they do not pose an absolute block to DNA cleavage in the context of stable higher-order complexes (G.C. Robinson, A.B.B. Burgin & A.M.S., unpublished results). The drawback of IntF is that the absence of the tyrosine decreases the accumulation and stability of some intermediate complexes (Segall, 1998), and does not lead to the accumulation of transient synaptic complexes between the substrates of integrative or excisive recombination pathways (Segall & Nash, 1993).

We have identified hexapeptides that efficiently block recombination at an early step (Cassell *et al.*, 2000, accompanying paper). Here, we describe the activities of two of these peptides, KWWCRW and KWWWRW, and show that they block DNA cleavage catalyzed by bacteriophage λ integrase. Elsewhere, we describe another set of peptides that trap the Holliday junction intermediate of Int-mediated recombination (G.C.C. & A.M.S., unpublished results).

Tyrosine recombinases conserve the energy of the cleavage event and use it for the ligation event. The same strategy is employed by DNA topoisomerases, which are divided into two major classes (Wang, 1985). The type I enzymes cut DNA one strand at a time, whereas the type II enzymes cut both DNA strands simultaneously. In turn, the type I enzymes are themselves subdivided into two subclasses, IA and IB, based on whether a free 3′ OH or a 5′ OH group is generated after nucleophillic attack. Because the tyrosine recombinases have a related mechanism and structural similarity to the eukaryotic type IB topoisomerases (Cheng *et al.*, 1998; Redinbo *et al.*, 1998, 1999; Stewart *et al.*, 1998), the inhibitory activity of the peptides was tested on the smallest and best studied of these enzymes, the vaccinia virus topoisomerase. For comparison, we also tested the inhibition by

Table 1. Summary of the four pathways of bacteriophage λ site-specific recombination

Pathway	Integration	Excision	Bent-L	Straight-L
att substrates	*attP*, *attB*	*attL*, *attR*	*attL* (*tenP′1*)[a]	*attL*
Int requirement	Y	Y	Y	Y
Bending protein requirement	IHF	IHF>HU, HMG1,2	IHF	Inhibitory
Xis requirement	Inhibitory	Y	N	N
supercoiling requirement	Y	N	N	N
Efficiency[b]	High	High	High	Low

[a] The bent-L pathway *in vivo* works equally well with wild-type *attL* or *attL tenP′1* substrates. However, the pathway works only with *attL tenP′1* substrates *in vitro* (Segall & Nash, 1996).

[b] High efficiency denotes >25 % conversion of substrates to products. Low efficiency denotes <10 % conversion of substrates to products.

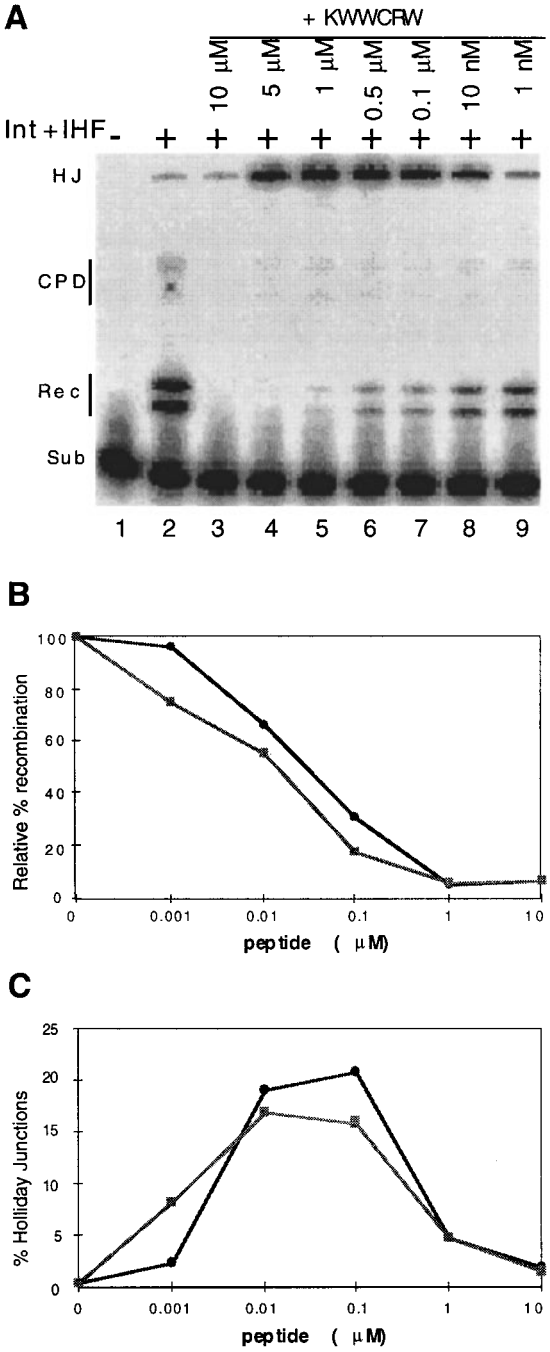

Figure 2. Effect of peptide inhibitors on bent-L recombination. (a) Recombination reactions were assembled as specified in Materials and Methods, containing one double end-labeled substrate (Sub) and a longer unlabeled substrate in the presence of 100 ng salmon sperm DNA. Recombinant products are labeled Rec, covalent protein-DNA intermediates are labeled CPD, and Holliday junctions are labeled HJ. Peptide was added at the specified concentrations. Recombination extents were normalized to the amount of recombination in untreated reactions and expressed as relative % recombination. (b) Comparison of the dose response titrations of peptide KWWCRW (filled circles) and peptide KWWWRW (filled squares) in bent-L recombination. The IC_{50} value for peptide KWWCRW is 0.02 μM, while for peptide KWWWRW it is roughly 0.04 μM. (c) The effect of peptide KWWCRW (filled circles) and peptide KWWWRW (filled squares) on accumulation of Holliday junctions during bent-L recombination. The % HJ were calculated as the fraction of total counts in each reaction × 100 %.

peptides of type IA and type II topoisomerases and of several restriction enzymes. We show that the peptides inhibit DNA cleavage with an effectiveness more or less related to the evolutionary similarity of these enzymes to each other: the peptides inhibit bacteriophage λ integrase best, vaccinia topoisomerase with somewhat less potency, are less potent still against the *Escherichia coli* type IA topoisomerase I, and are least potent against the type II T4 topoisomerase and restriction enzymes.

Results

Peptide inhibition of λ integrase

Several hexameric peptides which inhibit the Int-mediated bent-L recombination pathway were identified by screening synthetic peptide combinatorial libraries using a positional scanning strategy (Cassell *et al.*, 2000, accompanying paper; Pinilla *et al.*, 1998). Two related peptides, KWWCRW (peptide 59) and KWWWRW (peptide 56), showed the strongest phenotype. The effect of KWWCRW on the bent-L reaction is shown in Figure 2(a). At 10 μM peptide, recombination was inhibited completely without accumulation of intermediates. The concentration of peptide that inhibited recombination by 50 % (IC_{50}) was less than 0.1 μM (Figure 2(b)). At intermediate peptide concentrations (1-0.01 μM), the proteinase K-resistant Holliday junction accumulated as recombination gradually increased. At concentrations below 0.01 μM, recombination levels approached those of untreated reactions (Figure 2(a)). The accumulation of Holliday junctions was greatest at peptide concentrations which did not completely inhibit recombination (1-0.1 μM; Figure 2(b) *versus* (c)). The peptides did not increase the level of protein-DNA covalent intermediates (CPDs; see Figure 1), showing that the ligation event was unaffected. In fact, peptide concentrations that blocked recombination also inhibited formation of these CPDs (Figure 2(a), lane 2 *versus* lanes 3 and 4). These data suggest that the peptides inhibit Int-mediated DNA cleavage, and that the interaction of more than one peptide with the protein and/or DNA components of the system is necessary to inhibit recombination completely. Because each complete round of recombination involves four DNA cleavage events, a suboptimal number of peptides inhibits some, but not all, DNA cleavages, and Holliday junctions accumulate.

During peptide library deconvolution, we used the bent-L recombination pathway because it is efficient, it involves only Int and IHF, and it uses linear substrates (Table 1). We then tested whether

peptides KWWCRW and KWWWRW inhibit the integrative, excisive and straight-L recombination reactions. Although all pathways were affected, the potency of the peptides differed in each pathway (Figure 3). KWWCRW was most effective in bent-L recombination ($IC_{50} = 0.02$ μM), less effective in straight-L recombination ($IC_{50} = 0.06$ μM) and integration ($IC_{50} = 0.2$ μM), and least effective in excision ($IC_{50} = 1.1$ μM). KWWWRW had a very similar potency profile. Although Int is the agent of DNA cleavage in all four pathways, Int carries out cleavage within intermediate complexes with distinct, pathway-specific conformations (Segall & Nash, 1996). Because neither IHF nor Xis proteins are involved in the straight-L pathway, either DNA and/or Int must be the target of the peptides. However, order-of-addition experiments and titration experiments have not identified Int alone or DNA alone as the target (data not shown), suggesting instead that an Int-DNA complex is the target. Our data indicate either that Int interacts with its substrates in a somewhat different way in each recombination pathway, thus presenting a somewhat different target for the peptide, or that the target is the same in each pathway but the abundance of this target complex differs in each pathway (see below).

Do peptides KWWCRW and KWWWRW inhibit recombination by interfering with the formation of higher order complexes? The formation of intermediates in the bent-L pathway depends on Int contacting two different types of sites, the higher affinity arm sites and the lower affinity core sites flanking the loci of DNA cleavage and strand exchange (Figure 1). In an electrophoretic mobility shift assay, we found that both peptides interfered slightly with contacts between Int and its arm-binding sites (Figure 4(a), compare amount of free substrate in lanes 5 and 8 with that in lane 2; see below for discussion of the DNA shifted into the wells). To determine the effect of KWWCRW on interactions of Int with its core-binding sites, we assembled the recombination complexes, known as intasomes or unimolecular complexes (UMC), on an *attL* variant substrate with four mutations in the IHF-binding site, collectively known as QH′. These mutations prevent the specific binding of IHF to the QH′ sequence (Gardner & Nash, 1986), but still allow IHF to bind and bend DNA non-specifically (Segall *et al.*, 1994). In this situation, the appropriate complex can only be formed in the presence of Int and only when IHF binds in a ''pseudo sequence-specific'' manner and bends DNA at the appropriate site; this situation demands more stable Int-core interactions than are necessary when IHF binds and bends the *attL* site in a sequence-specific fashion (Segall *et al.*, 1994). The peptides did not interfere with formation of the Int/IHF/*attL-QH′* complex (Figure 4(b)), despite the peptide's effect on arm binding of Int. This suggested that the overall stability of the intasome suppressed the negative effect of the peptides on arm binding by Int. We then tested the assembly

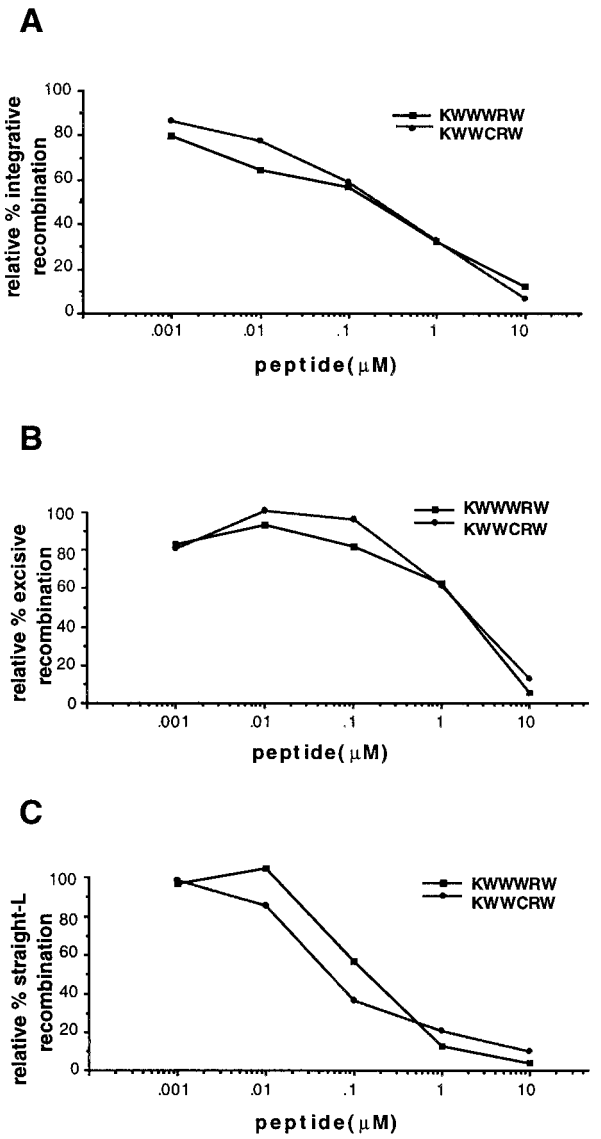

Figure 3. Effect of peptide KWWCRW (diamonds) and peptide KWWWRW (squares) on the remaining three pathways of Int-mediated recombination. Recombination extents were normalized to the amount of recombination in untreated reactions and expressed as relative % recombination. (a) Effect of peptides on integrative recombination (IC_{50} values for both peptides are roughly 0.2 μM). Reactions were assembled as for bent-L recombination except that they were incubated at room temperature; the substrates were a supercoiled plasmid (4.8 kb) carrying *attP* and ^{32}P-labeled 91 bp PCR fragment encoding *attB*. (b) Effect of peptides on excisive recombination (IC_{50} values for both peptides of about 1.1 μM) . The recombination substrates were PCR fragments encoding the *attL* (^{32}P-labeled) and *attR* sites. Reactions contained 50 nM Xis in addition to Int and IHF (37 nM), as well as 100 ng salmon sperm DNA, and were incubated at room temperature. (c) Effect of peptides on straight-L recombination (IC_{50} values for peptide KWWCRW is 0.06 μM, while for peptide KWWWRW is slightly over 0.1 μM). Reactions were assembled as for bent-L recombination except that they were incubated at room temperature; the substrates were two PCR fragments, one of which was ^{32}P-labeled and 187 bp, the other was unlabeled and 496 bp.

of bent-L pathway intermediates. At 10 μM peptide, all of the labeled DNA was shifted into the well (Figure 4(c), lanes 4 and 7; Figure 4(a), lanes 3 and 6). However, at lower peptide concentrations that still inhibited recombination (0.1-1 μM), intermediates were assembled normally. In fact, one of the intermediates, the bimolecular complex (BMC), accumulates substantially in the presence of the peptide (see also Figure 5). When this intermediate was analysed on a second, SDS-containing gel, it was found to contain Holliday junctions (data not shown). This agrees perfectly with our observations that suboptimal concentrations of peptide lead to accumulation of Holliday junctions (Figure 2). Both KWWCRW and KWWWRW appear to bind to DNA, although the reactions contain 100 ng of salmon sperm DNA in addition to the *att* substrates. The peptide shifts *att* site DNA even in the complete absence of Int (Figure 4(d)), confirming that KWWCRW interacts with DNA in a concentration-dependent fashion and in a manner that affects the mobility of the DNA much more drastically than expected for the size of the peptides.

We examined whether the inhibitory properties of the peptide were correlated with its DNA binding by testing the effect of increasing concentrations of salmon sperm DNA on the mobility and assembly of intermediates and on recombination. The results showed that the presence of 0.3 μg salmon sperm DNA concentration reversed the effect of 10 μM peptide concentration on the mobility of *att* intermediates (Figure 5(a)). However, the presence of 0.3-1 μg salmon sperm DNA did not reduce the peptide's ability to inhibit recombination (Figure 5(b)). We interpret these results to mean that the peptides either exhibit sequence-specific DNA binding or display a high level of affinity for some conformational feature specific to recombination intermediates.

Int, like its relative tyrosine recombinases, makes transient covalent protein-DNA complexes (CPDs) during the cleavage stage of the recombination reaction. While most of these complexes proceed through strand exchange and ligation, a small percentage of them do not and can be visualized on SDS-containing gels as proteinase K-sensitive species (e.g. Figure 2; Cassell *et al.*, 2000, accompanying paper). Since these complexes are the product of DNA cleavage by Int prior to strand exchange or ligation, we tested the effect of peptide KWWCRW on their formation. The peptide inhibited accumulation of both *attL* and *attR* CPDs by 65-75 % (data not shown), supporting our model that KWWCRW blocks recombination by interfering with the cleavage step of the reaction.

In order to test the specificity of peptide inhibition, we determined whether peptide KWWCRW affects the activity of a relative of the Int recombinase, namely the bacteriophage P1 Cre protein. Indeed, the peptide inhibits Cre-mediated recombination between two *lox* site substrates (G.C. & A.M.S., unpublished results). Based on these results, we asked whether the peptides inhibit enzymes with similar mechanisms of action that are less closely related to Int.

Peptide inhibition of vaccinia topoisomerase

Vaccinia virus topoisomerase, a prototypal type IB enzyme, is structurally and mechanistically similar to the tyrosine recombinases (Cheng *et al.*, 1998). The effects of the anti-Int peptides on the DNA relaxation activity of vaccinia topoisomerase are shown in Figure 6. The reaction mixtures contained the minimum amount of input topoisomerase that sufficed to relax the pUC19 DNA to completion in five minutes, as determined by end-point dilution in twofold increments (data not shown). Peptides KWWWRW and KWWCRW inhibited DNA relaxation in a concentration-dependent manner. Activity was abolished at 10-15 μM peptide and reduced by one-half at approximately 3-4 μM peptide. Two other aromatic hexapeptides, WCHYNY and WKHYNY, had no effect on DNA relaxation by vaccinia topoisomerase at peptide concentrations up to 42 μM (Figure 6, and data not shown). These latter two peptides appear to stabilize Holliday junctions but by a different mechanism than peptides KWWCRW or KWWWWRW (Cassell *et al.*, 2000, accompanying paper).

The catalytic cycle of vaccinia topoisomerase entails multiple steps: (i) non-covalent binding of enzyme to duplex DNA; (ii) scission of one strand with concomitant formation of a covalent DNA-(3′-phosphotyrosyl)-topoisomerase adduct; (iii) strand passage; and (iv) strand religation. Vaccinia topoisomerase displays stringent sequence specificity in DNA cleavage; it binds and forms a covalent adduct at sites containing the sequence 5′(C/T)CCTT↓ (Shuman & Prescott, 1990). This feature of the vaccinia enzyme facilitates analysis of the partial reactions using model substrates containing a single CCCTT cleavage site. ''Suicide'' substrates have been especially useful for studying the cleavage reaction (first transesterification) under single-turnover conditions. An example of such a substrate is shown in Figure 7. Covalent adduct formation is accompanied by spontaneous dissociation of the 3′ fragment of the cleaved strand from the topoisomerase-DNA complex, which leaves a 18-nucleotide single-strand tail on the non-cleaved strand. With no readily available acceptor for religation, the topoisomerase is covalently trapped on the DNA. The single-turnover reaction is complete within 15 seconds at 37 °C. The yield of covalent adduct is proportional to input topoisomerase when DNA is in excess and the reaction is near-quantitative at saturating enzyme. Peptide effects were evaluated at enzyme concentrations sufficient to cleave 60-70 % of the input substrate in ten seconds. In the experiment shown in Figure 7, the peptides were pre-incubated with DNA prior to adding topoisomerase. Peptides KWWWRW and KWWCRW, which

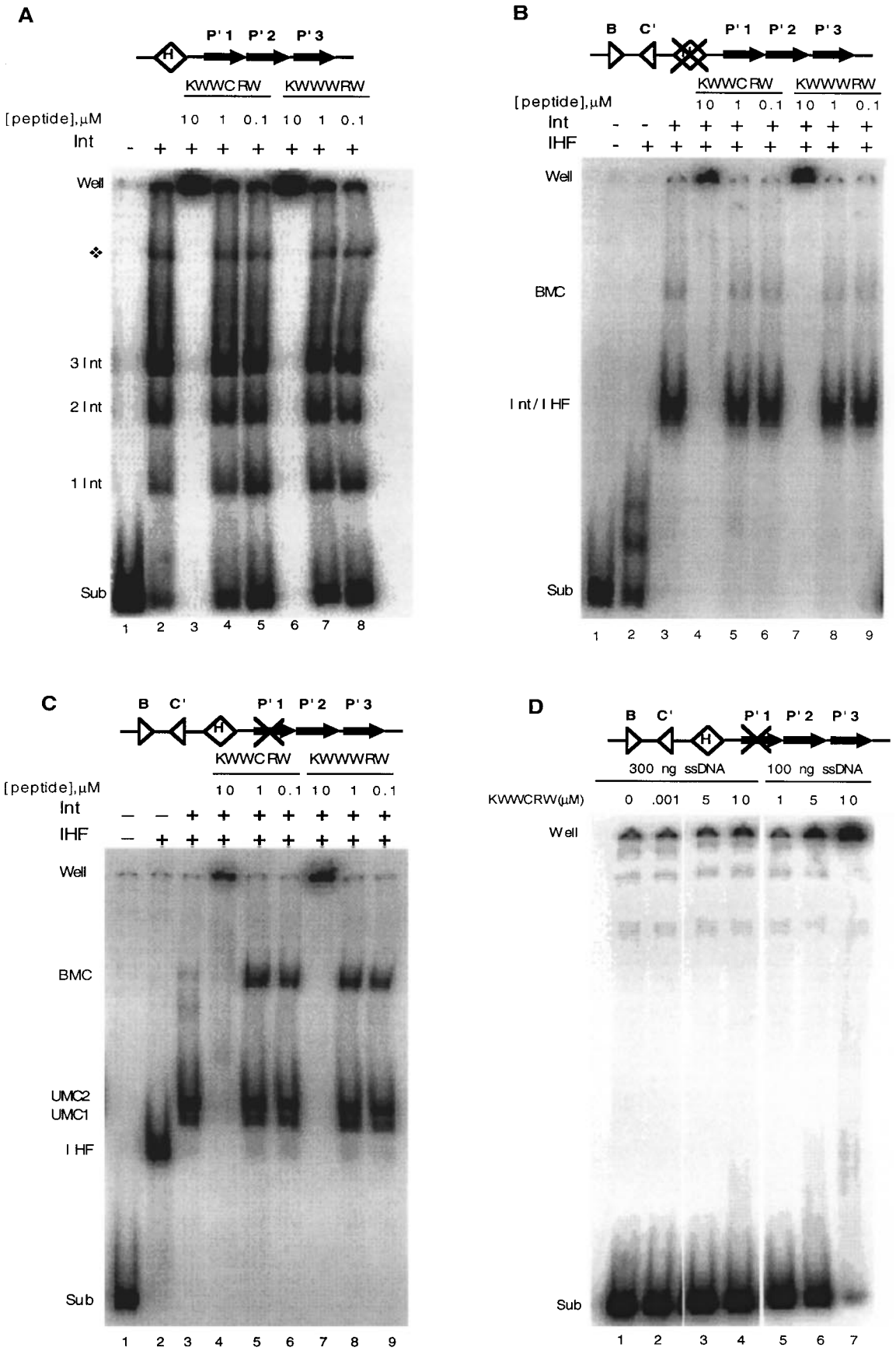

Figure 4 (*legend opposite*)

blocked DNA relaxation, were potent dose-dependent inhibitors of covalent adduct formation (99 % inhibition at 1.6 to 1.8 μM; IC_{50} at ~0.5 μM), whereas peptides WKHYNY and WCHYNY did not inhibit transesterification (Figure 7, and data not shown). Inhibition of DNA cleavage by KWWWRW and KWWCRW as a function of peptide concentration did not change when the order of addition was varied, e.g. when topoisomerase was pre-incubated with peptides prior to the addition of the DNA substrate (data not shown). Kinetic analysis showed that the KWWWRW and KWWCRW peptides slowed the rate of transesterification. The magnitude of the rate effect increased sharply as the peptide concentrations were increased from 0.64 to 1.6 μM and 0.72 to 1.8 μM, respectively (Figure 8).

To test whether the mechanism of topoisomerase inhibition necessitates direct interaction between the peptides and the DNA, we examined the effect of ionic strength on potency of the peptides. The potency of the KWWCRW peptide as an inhibitor of DNA cleavage by vaccinia topoisomerase was sensitive to changes in the ionic strength of the reaction mixture. Inclusion of 100 mM NaCl in the cleavage reactions resulted in a shift to the right in the peptide inhibition curve (Figure 9). Whereas 0.7 μM peptide reduced covalent adduct formation by 90 % in the absence of added salt, the same concentration of peptide inhibited cleavage by only 40 % in the presence of 100 mM NaCl. We noted a similar decrement in the potency of the KWWCRW and KWWWRW peptides in inhibiting relaxation for supercoiled plasmid DNA by vaccinia topoisomerase when the relaxation reaction mixtures were supplemented with 100 mM NaCl (data not shown). These results suggest that the peptide probably interacts with DNA as part of its inhibitory mechanism.

Peptide inhibition of suicide cleavage can be explained by either of the following: (i) the peptides directly inhibit transesterification, or (ii) the peptides block non-covalent binding of topoisomerase to the DNA. We sought to address this issue by circumventing the DNA binding step, i.e. by studying the ability of topoisomerase already bound covalently to the suicide substrate to catalyze religation to an acceptor DNA provided in *trans* (Sekiguchi *et al.*, 1997). The acceptor strand was a 5′OH-terminated 18-mer complementary to the 5′ tail of the ''donor'' complex. The religation product was a 30-mer that was resolved electrophoretically from the input 18-mer strand. Single-turnover religation was fast and efficient; 80 % of the input substrate was religated to the exogenous acceptor in ten seconds (data not shown). Treatment of the covalently bound topoisomerase for five minutes with up to 40 μM of the KWWCRW or KWWWRW peptides (concentrations that abrogated the suicide cleavage reaction) prior to the addition of the acceptor strand had no discernible effect on strand religation (not shown). Thus, the peptides did not inhibit transesterification chemistry; rather, they blocked a step unique to the forward cleavage reaction.

To test whether the peptide interferes with the non-covalent association of topoisomerase with the DNA, we assayed the effects of the peptides on the binding of vaccinia topoisomerase to a radiolabeled 60-bp duplex DNA containing a single central CCCTT recognition site. In contrast to the suicide substrate, for which all bound enzymes are trapped in the covalent state, only about 20 % of the fully double-stranded DNA molecules that are bound will be linked covalently to the protein (Wittschieben & Shuman, 1997). Hence this gel shift assay largely reflects the non-covalent binding of enzyme to the DNA ligand. The most instructive finding was that concentrations of the KWWCRW peptide sufficient to block covalent adduct formation (0.72-1.8 μM peptide; see Figure 7) did not inhibit formation of the non-covalent topoisomerase-DNA complex (Figure 10). At higher concentrations (3.6-7.2 μM), peptide KWWCRW changed the mobility of the labeled DNA and resulted in its retention in the sample well (Figure 10). A partial shift to the well was observed at 1.8 μM KWWCRW and lower concentrations of peptide (0.32-0.72 μM) had no effect on the mobility of the topoisomerase-DNA complex. The concentration-dependent shift of the 60-bp DNA to the sample well by the KWWCRW peptide does not depend

Figure 4. Interaction of peptides with recombination intermediates and DNA substrates. (a) Peptide effects on interactions between Int and arm binding sites. The labeled substrate (2 nM in the reaction) is a PCR fragment including the IHF and three tandem arm sites of *attL*. Assays were performed in the presence of Int only (50 nM) and 300 ng salmon sperm DNA and incubated 30 minutes at room temperature before being separated on a 7.5 % polyacrylamide native gel at 165V. (b) Peptide effects on contacts between Int and the core of *attL QH′*. Because the IHF binding site is mutated, IHF binds and bends DNA pseudo-specifically only in the presence of Int (Segall *et al.*, 1994). To overcome the loss of specific binding energy and form an intasome complex, Int must make stable contacts with the core. Reactions contained 2 nM *attL QH′* substrate (described in the text), 50 nM Int, 37 nM IHF, and 100 ng salmon sperm DNA, and were incubated 30 minutes at room temperature before electrophoresis on a native 5 % polyacrylamide gel (165V). (c) Peptide effects on assembly of bent-L recombination intermediates. Reactions were assembled as for recombination reactions (see Materials and Methods) containing 2 nM of the labeled *attL tenP′1* substrate (wild-type IHF site but mutant P′1 arm site to prevent Int binding; see the text). In the presence of both Int and IHF, 2 UMC complexes are assembled which contain a single *att* molecule, and a BMC complex containing 2 *att* molecules. (d) Peptide KWWCRW (peptide 59) effect on mobility of *attL tenP′1* in the presence of 100 or 300 ng salmon sperm DNA in the absence of Int or IHF. All reactions also contained 60 mM final KCl.

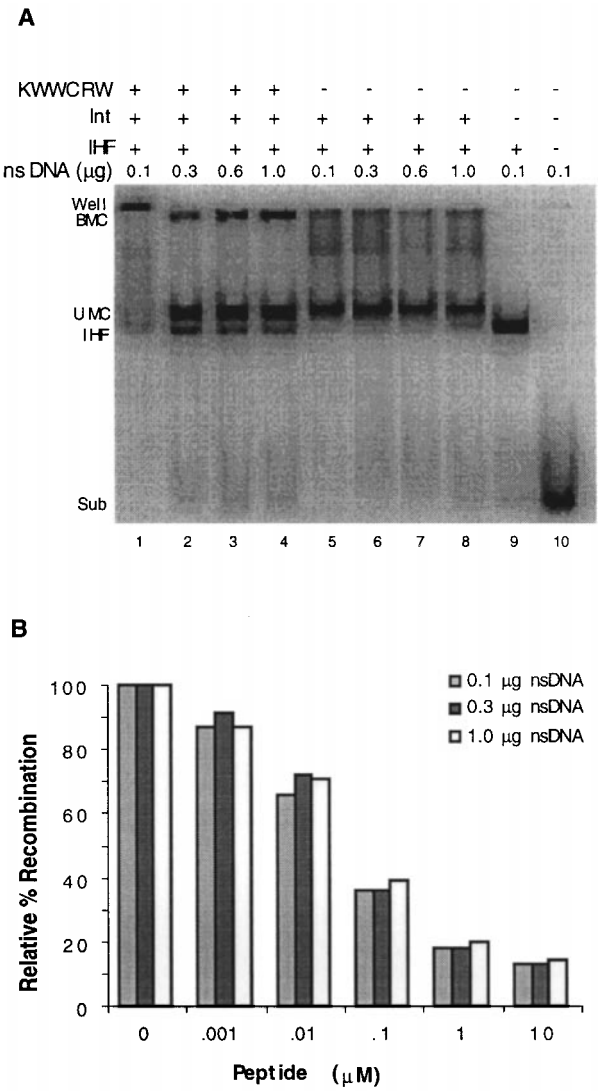

Figure 5. Effect of salmon sperm DNA concentration on the effects of peptide inhibitors. (a) Peptide effects on assembly of recombination intermediates in the presence of increasing concentration of salmon sperm DNA. (b) Peptide KWWCRW effect on bent-L recombination in the presence of increasing concentration of salmon sperm DNA.

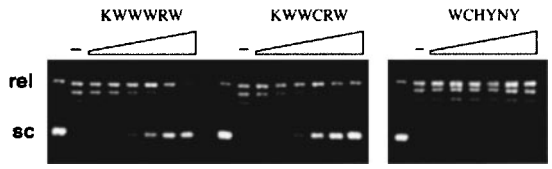

Figure 6. Peptide inhibition of DNA relaxation catalyzed by vaccinia topoisomerase. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 2.5 mM EDTA, 0.3 μg of pUC19 DNA, 0.5 pmol of vaccinia topoisomerase, and increasing concentration of the indicated hexapeptides were incubated for five minutes at 37°C. The reactions were quenched by addition of a stop solution containing SDS, glycerol, and bromphenol blue. The products were analyzed by electrophoresis through a 1% horizontal agarose gel in TG buffer (50 mM Tris, 150 mM glycine). The gel was stained with a solution of 0.5 mg/ml ethidium bromide, destained in water, and photographed under short-wave UV illumination. A reaction containing supercoiled DNA without added topoisomerase is shown in the leftmost lane of each peptide titration series. A mixture containing topoisomerase and no added peptide in shown in lane (−). The concentrations of the added peptides (proceeding from left to right in each series) were as follows: 0.64, 1.6, 3.2, 6.4, and 12.8 μM KWWWRW; 0.72, 1.8, 3.6, 7.2, and 14.4 mM KWWCRW; 0.84, 2.1, 4.2, 8.4, 16.8, and 42 mM WCHYNY; 0.83, 2.1, 4.2, 8.3, 16.8, and 42 μM WKHYNY (data for this last peptide not shown).

on the presence of topoisomerase (Figure 10; DNA alone). We surmise that the peptide binds directly to the DNA and alters its structure, although no discrete peptide-DNA complexes of intermediate mobility were seen in the course of the peptide titration. Apparently the peptide binds to the topoisomerase-DNA complex at these concentrations (and presumably to free DNA also) in such a way as to inhibit topoisomerase cleavage, but without causing the more drastic alteration in DNA structure that results in wholesale shift to the well at higher levels of peptide concentrations. The WCHYNY peptide, which did not inhibit vaccinia topoisomerase but which does lead to accumulation of Holliday junctions (see Cassell *et al*., 2000, accompanying paper), had no effect on the mobility of the topoisomerase-DNA complex (Figure 10).

Peptide inhibition of *Escherichia coli* DNA topoisomerase I

E. coli topoisomerase I (TopA) exemplifies the type IA topoisomerase family. Type IA enzymes are mechanistically and structurally unrelated to the topoisomerase IB/tyrosine recombinase superfamily of DNA strand transferases. Nonetheless, the relaxation of supercoiled DNA by *E. coli* TopA was inhibited in a concentration-dependent manner by the KWWWRW and KWWCRW peptides. Activity was abolished at 15-42 μM peptide and reduced by one-half at approximately 7-10 μM peptide (Figure 11). The other aromatic hexapeptides, WCHYNY and WKHYNY, had no effect on DNA relaxation by *E. coli* topoisomerase I at peptide concentrations up to 42 μM (Figure 11 and data not shown). The specificity of peptide inhibition of DNA relaxation was similar for type IB and type IA topoisomerases, but the inhibitory peptides were about twice as potent on a molar basis against the type IB topoisomerase.

Inhibition of type II topoisomerase and restriction endonucleases

We further challenged the specificity of action of peptide KWWCRW by testing its effect on bacteriophage T4 topoisomerase, a type II enzyme.

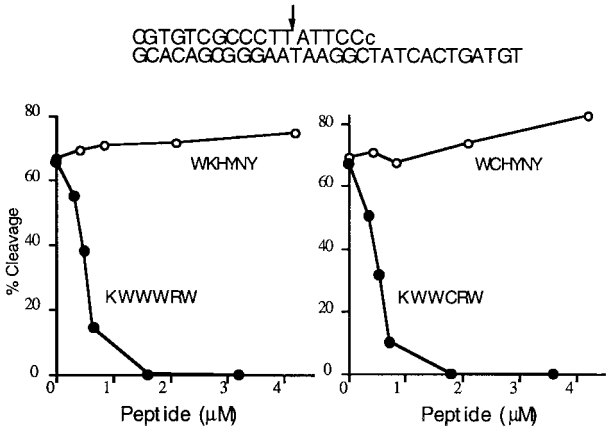

Figure 7. Peptide inhibition of single-turnover DNA cleavage catalyzed by vaccinia topoisomerase. The structure of the CCCTT-containing suicide substrate is shown, with the cleavage site is indicated by the arrow. The DNA was (5′-^{32}P)-labeled on the scissile strand. Cleavage reaction mixtures (20 μl) contained 50 mM Tris-HCl (pH 7.5), 0.1 pmol of 18-mer/30-mer DNA substrate, 0.5 pmol of vaccinia topoisomerase, and peptides as specified. Mixtures containing buffer and DNA were pre-incubated with the peptides for ten minutes at 37 °C in the absence of topoisomerase. The cleavage reactions were initiated by adding topoisomerase and quenched after ten seconds at 37 °C by adding SDS to 1% final concentration. The denatured samples were electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. The extent of covalent adduct formation (expressed as the percentage of input labeled DNA transferred to the topoisomerase polypeptide) was quantified by scanning the gel with a PhosphorImager and is plotted as a function of the concentration of peptide in the reaction mixtures. (a) Titration of KWWWRW and WKHYNY. (b) Titration of KWWCRW and WCHYNY.

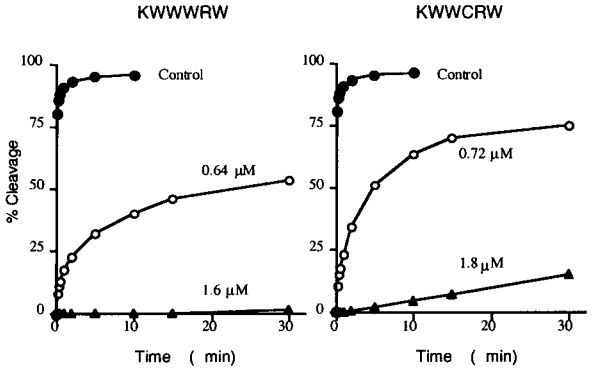

Figure 8. Peptide effects on the kinetics of DNA cleavage by vaccinia topoisomerase. Reaction mixtures containing (per 20 μl) 50 mM Tris-HCl (pH 7.5), 0.1 pmol of 18-mer/30-mer DNA substrate, 0.5 pmol of vaccinia topoisomerase, and peptides as specified were incubated at 37 °C. The reactions were initiated by the addition of enzyme to DNA (control) or to the preincubated DNA/peptide mixture. Aliquots (20 μl) were withdrawn at the times indicated and quenched immediately with SDS. Covalent adduct formation is plotted as a function of time.

Indeed, KWWCRW inhibited T4 topoisomerase-induced DNA relaxation with an IC_{50} of 40 μM and blocked it completely at 100 μM, while the similarly aromatic peptide WKHYNY had no effect on relaxation at 100 μM (Figure 12). Because KWWCRW binds DNA, we also tested its effect on the activity of several restriction enzymes with unique sites in pUC19 (Figure 13). Although each enzyme's recognition sequence contains a different distribution of A·T and G·C base-pairs, all of the enzymes were inhibited with a similar IC_{50}, roughly 40 μM. These results indicate that the peptide's DNA-binding property may interfere relatively non-specifically with the activities of several DNA-cutting enzymes. A summary of IC_{50} values for the inhibition of DNA cleaving enzymes discussed here is given in Table 2.

Discussion

The detailed analysis of biochemical reactions depends on the ability to trap and study reaction intermediates. This has been particularly difficult in the case of reactions catalyzed by tyrosine recombinases, which are very efficient, freely reversible, and do not require any high-energy cofactors. Cellular type IB topoisomerases are mechanistically similar to the tyrosine recombinases, and the analysis of their reactions with DNA has been aided by the availability of inhibitors such as camptothecin, which stabilizes a covalent reaction intermediate (Rothenberg, 1997). Such mechanistic inhibitors have not been available for the tyrosine recombinases or for the vaccinia virus topoisomerase. While netropsin, a minor groove-binding compound, does block recombination by competing with Int and with IHF for interactions with their respective DNA binding sites, it has not been useful in trapping reaction intermediates (R. Moision & A.M.S., unpublished results).

Here, we have characterized two peptide inhibitors of DNA cleavage by λ integrase. These inhibitors were identified using a deconvolution process of combinatorial peptide libraries (Cassell *et al.*, 2000, accompanying paper; Pinilla *et al.*, 1998) and represent the first peptide inhibitors of tyrosine recombinases. The potency of the peptides differs for the different pathways of Int-mediated recombination (Table 2). In the case of the bent-L and straight-L pathways, the substrates are identical at the loci of strand cleavage (and elsewhere except for three-base substitutions in the P′1 arm binding site), yet the peptides inhibit the bent-L pathway threefold more efficiently than the straight-L pathway. The peptides inhibit integrative recombination with a somewhat higher IC_{50}, 0.2 μM; the *attP* substrate has additional DNA binding sequences important for recombination and is supercoiled, while *attB* contains only the core sequences, which

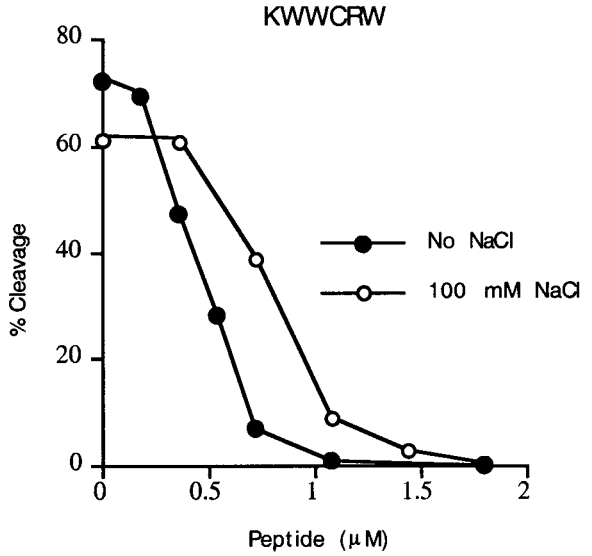

Figure 9. Salt diminishes peptide potency in inhibiting vaccinia topoisomerase. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 0.1 pmol of 18-mer/30-mer substrate, 0.5 pmol of vaccinia topoisomerase, KWWCRW peptide as specified, and either 100 mM NaCl or no added NaCl were incubated for ten seconds at 37 °C. The extent of covalent adduct formation is plotted as a function of peptide concentration.

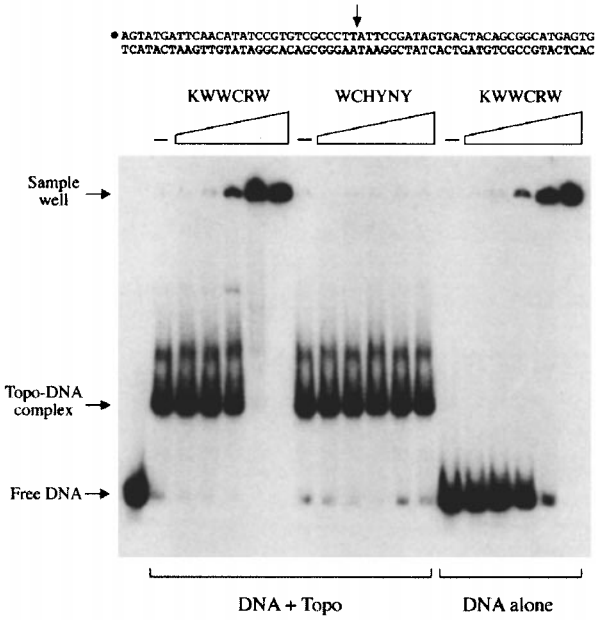

Figure 10. Peptide effects on DNA binding to vaccinia topoisomerase. The structure of the 60-bp CCCTT-containing DNA is shown with the site of covalent adduct formation indicated by an arrow. The (5′-^{32}P)-label on the scissile strand is denoted by a dot. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 0.5 pmol of 60-bp DNA, and increasing amounts of peptides as specified, and either 1 pmol of vaccinia topoisomerase (DNA + Topo) or no added protein (DNA alone) were incubated for five minutes at 37 °C. The reaction mixtures were incubated at 37 °C for five minutes. Glycerol was added to 8% (v/v) and the samples were electrophoresed through a 6% native polyacrylamide gel in 0.25 × TBE at 100 V for three hours. An autoradiograph of the gel is shown. The concentrations of the added peptides (proceeding from left to right in each series) were as follows: 0.36, 0.72, 1.8, 3.6, and 7.2 μM KWWCRW; 0.42, 0.84, 2.1, 4.2, and 8.4 μM WCHYNY. Control reaction mixtures containing no added peptide are shown in lanes (−).

are almost identical among all four Int substrates. Excisive recombination substrates are very closely related to integrative recombination substrates, but the distribution of protein binding sites along the DNA is different (Figure 1). Moreover, an additional accessory protein, Xis, is necessary for excision. This pathway is inhibited with an IC_{50} of 1.1 μM. It appears unlikely that the minor differences in DNA sequence underlie the difference in IC_{50} values in the four pathways. We conclude that the difference in potency of peptides KWWCRW and KWWWRW in each pathway reflects differences among the pathways in the interactions of Int with the loci of strand cleavage. Int interactions could vary due to a combination of architectural, kinetic, and stability factors. Furthermore, the rate-limiting step may be distinct for each recombination pathway, and thus the mechanistic step targeted by the peptide may not have an equal effect in all of the pathways. The basis of differences between the inhibitory potency of the peptides in each pathway is being investigated, and libraries are being screened for active peptides using the excision pathway.

We do not yet know the mechanism by which the peptides inhibit DNA cleavage, nor the exact nature of their target. Although the peptides clearly bind and probably deform double-stranded DNA into a conformation that prevents it from entering a polyacrylamide gel (Figures 4 and 10), peptide inhibition of Int is resistant to as much as 1 μg of non-specific competitor DNA (Figure 5). This suggests that the target of the peptide is a specific complex of enzyme with its substrate, or requires that the DNA substrate be in some way deformed by Int. Although the peptide does slightly decrease Int binding to its arm sites, it does not prevent Int from making stable contacts with the core sites in the context of either early (UMC species) or synaptic (BMC species; Figure 4) recombination intermediates. Therefore, the peptide more specifically targets interactions between Int and DNA which are necessary for DNA cleavage. Indeed, cleavage of both excision substrates is inhibited by peptide KWWCRW (data not shown). One possibility is that Int, like Cre (Guo *et al.*, 1999) locally kinks the DNA double helix at the site of cleavage prior to nucleophilic attack, resulting in the unstacking of two base-pairs. This possibility is supported by two pieces of evidence: (i) the peptide has a somewhat higher affinity for

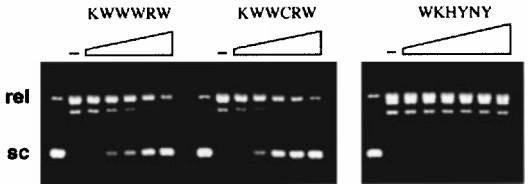

Figure 11. Peptide inhibition of DNA relaxation catalyzed by *E. coli* topoisomerase I. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.3 μg of pUC19 DNA, 0.1 pmol of *E. coli* topoisomerase I, and increasing concentration of the indicated hexapeptides were incubated for five minutes at 37°C. The reaction products were analyzed by electrophoresis through a 1% horizontal agarose gel. A photograph of the ethidium bromide-stained gel is shown. A reaction containing supercoiled DNA without added topoisomerase is shown in the leftmost lane of each peptide titration series. A mixture containing topoisomerase and no added peptide in shown in lane (−). The concentrations of the added peptides (proceeding from left to right in each series) were as follows: 0.64, 1.6, 3.2, 6.4, 12.8 and 32 μM KWWWRW; 0.72, 1.8, 3.6, 7.2, 14.4 and 36 μM KWWCRW; 0.84, 2.1, 4.2, 8.4, 16.8, and 42 μM WCHYNY (data not shown); 0.83, 2.1, 4.2, 8.3, 16.8, and 42 μM WKHYNY.

single-stranded than for double-stranded DNA (data not shown); and (ii) Int makes the bases at the loci of strand cleavage hypersensitive to dimethyl sulfate (Segall, 1998), which modifies single-stranded DNA more efficiently than double-stranded DNA. This model and the implication of an additional intermediate step in the mechanism of Int-mediated recombination is being tested in detail.

The KWWCRW and KWWWRW peptides also inhibit a related tyrosine recombinase, the Cre enzyme of bacteriophage P1, as well as the more distantly related but mechanistically similar vaccinia virus topoisomerase. Although the peptides were most effective at inhibiting the pathway with which we screened them, the IC_{50} of the peptides for the vaccinia topoisomerase is in the same range as the IC_{50} for Int in integration and excision (Table 2). Moreover, the peptide inhibits DNA cleavage even at concentrations which have no effect on the non-covalent complex between the vaccinia topoisomerase and its DNA substrate. Thus, as in the case of Int, the mechanism of cleavage inhibition appears specific to enzyme-substrate interactions necessary for catalysis.

As might be expected for peptide inhibitors that bind to DNA, the KWWCRW and KWWWRW peptides are not entirely specific to enzymes that employ a type IB topoisomerase mechanism. For example, they inhibit, albeit with a lower potency, the action ot *E. coli* topoisomerase I, an enzyme that cleaves DNA one strand at a time *via* a transient 5′-phosphotyrosine linkage and leaves a free 3′ OH group (Wang, 1996). This enzyme has been shown to bind preferentially to single-stranded DNA, and may cleave DNA *via* a single-stranded DNA intermediate. In addition, the two peptides inhibit the T4 topoisomerase, a type II enzyme that also uses a tyrosine in a nucleophilic attack on the DNA phosphodiester backbone, but with a much reduced potency (an IC_{50} of 40 μM, which is as much as 2000-fold lower than the potency of Int inhibition; Table 2).

One possible explanation for the lower level of potency of the peptides for the T4 topoisomerase and the *E. coli* topoisomerase I is that these topoisomerases have multiple target sites in the plasmid substrates, at which they act with similar efficiency; the higher IC_{50} may simply reflect the necessity for more peptides to interact with all of the available target sites. In contrast, DNA cleavage for Int, Cre, and vaccinia topoisomerase was assayed on substrates in which a single target site was available. Therefore, we tested the inhibitory effect of the peptides on cleavage by several restriction enzymes, each of which has a single target sequence in pUC19. Each of these enzymes was inhibited with a similar IC_{50} (Table 2), despite the

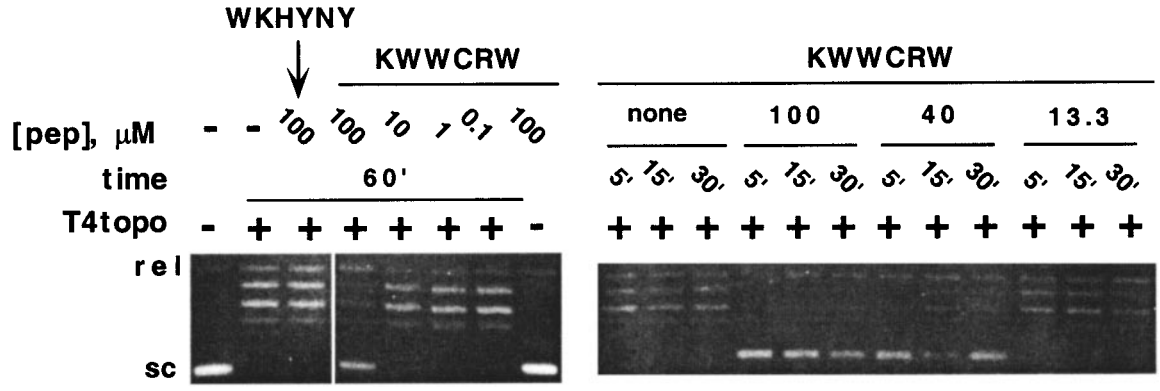

Figure 12. Peptide inhibition of DNA relaxation catalyzed by T4 topoisomerase. Peptide KWWCRW (59) or WKHYNY (52) were added at the specified concentration to 20 μl reactions containing 0.2 μg pUC19 and 60 ng T4 topoisomerase. Dose-response assays are shown in the left panel while the effect of different peptide doses on the kinetics of relaxation is shown on the right.

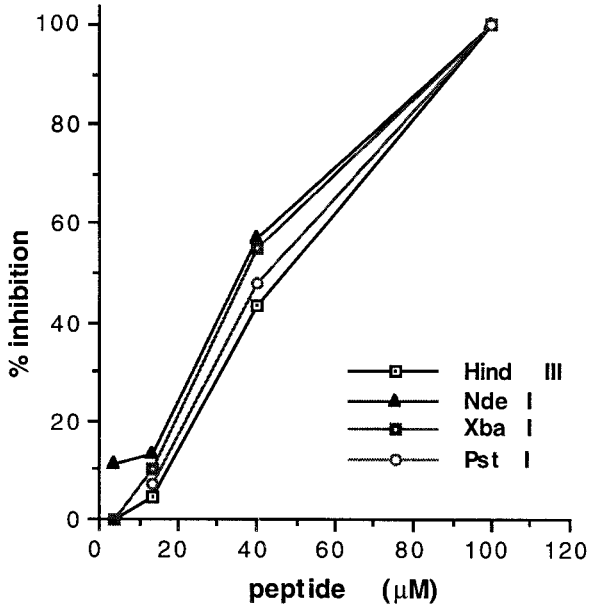

Figure 13. Peptide inhibition of four restriction enzymes. Reactions contained 0.2 μg of pUC19 DNA and 10 units of each of the specified restriction enzyme (NEB). Reactions were incubated for 30 minutes at 37 °C, stopped with SDS-containing loading dye and electrophoresed on 0.8 % agarose gels. The DNA was visualized with EtBR, photographed, scanned and quantitated with NIH Image v. 1.55. The percentage inhibition was calculated with respect to digestion in parallel reactions without peptide.

fact that their restriction sites have different A/T *versus* G/C content and different distribution of the A/T *versus* G/C base-pairs. Thus, the peptides are significantly less potent against either the T4 topoisomerase or the restriction endonucleases, and may inhibit these enzymes as a consequence of relatively non-specific interactions with DNA. We propose that the peptides inhibit DNA cleavage in two distinct ways: by interacting specifically with enzyme-DNA intermediates in the case of the tyrosine recombinases and the vaccinia type Ib topoisomerase (and perhaps less efficiently in the case of the *E. coli* type Ia topoisomerase), and by interacting non-specifically with DNA in the case of the T4 topoisomerase and restriction enzymes.

Our study has shown that specific hexameric peptides are potent inhibitors of DNA cleavage by tyrosine recombinases. The peptides are useful new tools for the analysis of the mechanism of site-specific recombination (here; G.C. & A.M.S., unpublished results). In addition, these peptides inhibit DNA cleavage by the vaccinia type I topoisomerase. This result shows that site-specific recombination can be used effectively as a screen for inhibitors against enzymes with related biochemical mechanisms. Such approaches should continue to be useful as well-studied reactions by prokaryotic enzymes can be used to screen inhibitors of structurally and mechanistically related eukaryotic enzymes.

Table 2. Summary of IC_{50} values for KWWCRW

Protein	IC_{50} (μM)
Integrase	
Bent-L	0.02
Straight-L	0.06
Integration	0.3
Excision	1.1
Vaccinia topoisomerase (type Ib)	0.5[a] (3.5)
E. coli topoisomerase I (type Ia)	8
T4 topoisomerase (type II)	40
*Hin*dIII[b] (AAGCTT)	48
*Nde*I (CATATG)	37
*Pst*I (CTGCAG)	44
*Xba*I (TCTAGA)	37

[a] IC_{50} for DNA cleavage is given, with the IC_{50} for plasmid relaxation in parentheses. In the plasmid relaxation assay, most of the plasmid DNA can be considered non-specific competitor DNA; this ''extra'' DNA is absent in the DNA cleavage assay (see Figure 7).

[b] The sequence of the recognition sites for each restriction enzyme is given in parentheses.

Materials and Methods

Proteins

Purified Int was the generous gift of C. Robertson and H. Nash (NIH), and of J. Hartley (Gibco BRL Life Technologies Inc.). Purified IHF was the generous gift of S.-W. Yang and H. Nash (NIH), while purified Xis was the generous gift of C. Robertson and H. Nash (NIH). HU was purified as described (Segall *et al*., 1996).

Vaccinia topoisomerase was expressed in *Escherichia coli* BL21 cells infected with bacteriophage λCE6 and then purified from a soluble bacterial lysate by phosphocellulose column chromatography (Shuman *et al*., 1988). The protein concentration of the phosphocellulose preparation was determined by using the dye-binding method (BioRad) with bovine serum albumin as the standard.

T4 topoisomerase was the generous gift of K. Kreuzer (Duke University). *E. coli* topoisomerase I was the generous gift of K. Marians (Memorial Sloan-Kettering Cancer Center). Cre protein and *lox* recombination substrates were generously provided by Alex Burgin. Restriction enzymes, VENT polymerase, and T4 polynucleotide kinase were purchased from New England BioLabs. [γ-^{32}P]ATP was purchased from New England Nuclear.

DNA substrates for Int and T4 topoisomerase assays

Linear substrates for site-specific recombination or mobility shift assays were synthesized by PCR using plasmids with cloned *attB*, *attL*, *attLtenP′1*, *attL-QH′*, or *attR* sites and labeled at the 5′ end with [γ-^{32}P]ATP using T4 polynucleotide kinase as described (Segall *et al*., 1994). Supercoiled pUC19 for relaxation assays by T4 topoisomerase and pHN894 containing the *attP* substrate for integration were isolated from DH5α cells using the Qiagen Midi plasmid purification kit (Qiagen).

DNA substrates for vaccinia topoisomerase

DNA oligonucleotides were 5′ end-labeled by enzymatic phosphorylation in the presence of [γ-^{32}P]ATP and T4 polynucleotide kinase, then purified by preparative electrophoresis through a 15% polyacrylamide gel containing TBE (90 mM Tris-borate, 2.5 mM EDTA). The labeled oligonucleotides were eluted from an excised gel slice and then hybridized to unlabeled complementary oligonucleotide(s) as specified in the Figure legends. Annealing reaction mixtures containing 0.2 M NaCl and oligonucleotides as specified were heated to 70°C and then slow-cooled to 22°C. The hybridized DNAs were stored at 4°C.

Int assays

Site-specific recombination and gel mobility shift assays were performed as described (Segall, 1998). Briefly, reactions were performed in a total volume of 10 or 20 μl and typically contained 1-2 nM radiolabeled *att* site as specified, 4 nM unlabeled *att* site, 100-300 ng salmon sperm DNA as nonspecific competitor, 44 mM Tris-HCl (pH 8.0), 60 mM KCl, 0.05 mg/ml bovine serum albumin, 7 mM Tris-borate (pH 8.9), 5 mM spermidine, 1.3 mM EDTA, and 14.6% (v/v) glycerol. Any deviation from this formulation is noted in the Figure legends. Gel shift reactions were incubated for 90 minutes at 37°C, layered without loading dyes onto 5% native polyacrylamide gel (29 acrylamide:1 bis-acrylamide) and electrophoresed in 0.5× Tris-borate EDTA buffer. Recombination reactions were stopped with 0.2× volume of 2% SDS, layered onto 5% polyacrylamide Tris/SDS gels, and electrophoresed in 1× Tris Tricine SDS buffer at 100 mA (Segall, 1998). Dried gels were visualized and quantified using a Molecular Dynamics PhosphorImager.

Restriction enzyme assays

Restriction digests were performed as specified and the products were separated on 0.8% agarose gels electrophoresed at 80-90V in 1× Tris-borate EDTA buffer. Gels were photographed, scanned and quantitated using NIH Image v.1.55, as recommended in the instruction manual.

T4 topoisomerase assays

Reactions were performed as decribed (Huff & Kreuzer, 1990) using 30 or 60 ng of enzyme and 200 ng of supercoiled pUC19 per reaction. The products were electrophoresed on 0.8% agarose gels at 40V in 0.5× Tris-borate EDTA buffer for about six hours. The gel was then stained with EtBr for viewing.

Peptides

Peptides were synthesized with a C-terminal amide group using TBOC-protected amino acid residues (Pinilla *et al*., 1998), followed by HPLC-purification, at Torrey Pines Institute for Molecular Studies. The molar concentrations of the peptides KWWWRW, KWWCRW, WCHYNY, and WKHYNY were calculated from the absorbance at 280 nm at neutral pH using the extinction coefficients of 1.4×10^3 M^{-1} for tyrosine and 5.6×10^3 M^{-1} for tryptophan.

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