Reversible Inhibitors of λ Integrase-mediated Recombination Efficiently Trap Holliday Junction Intermediates and Form the Basis of a Novel Assay for Junction Resolution*

Received for publication, August 25, 2003, and in revised form, November 13, 2003 Published, JBC Papers in Press, November 18, 2003, DOI 10.1074/jbc.M309361200

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The bacteriophage λ integrase catalyzes four site-specific recombination pathways with distinct protein and DNA requirements and nucleoprotein intermediates. Some of these intermediates are very transient and difficult to obtain in significant amounts, due to the high efficiency and processivity of integrase, the lack of requirements for external energy factors or metal ions, and the highly reversible nature of each of the intermediates. We have previously used mixture-based combinatorial libraries to identify hexapeptides that trap 40–60% of recombination substrates at the Holliday junction stage of the reaction. These inhibitors discriminate between the four pathways, blocking one of them (bent-L recombination) more severely than the others and blocking the excision pathway least. We presume that these differences reflect specific conformational differences of the nucleoprotein intermediates in each pathway. We have now identified new inhibitors of the excision pathway. One of these, WRWYCR, is over 50-fold more potent at inhibiting excision than the previously identified peptides. This peptide stably traps Holliday junction complexes in all recombination pathways mediated by integrase as well as Cre. This finding and other data presented indicate that the peptide’s target is a common feature shared by the Holliday junction complexes assembled by tyrosine recombinases. We have taken advantage of reversible inhibition by the active peptides to develop a new assay for Holliday junction resolution. This assay is particularly useful for determining junction resolution rates in cases where complexes directly assembled on junction substrates undergo little or no catalysis.

Tyrosine recombinases catalyze site-specific recombination between partner sequences via two rounds of single-stranded cleavage, exchange, and ligation (see Fig. 1) (1, 2). Because catalysis on the “top” strands of each substrate is separated temporally from catalysis on the “bottom” strands, a Holliday junction is made during the first round of catalysis and resolved during the second round. Normally, the Holliday junction is extremely transient, and no more than ~2% of substrates accumulate at this stage, making this intermediate recalcitrant to study. We have previously identified several hexapeptides that trap substrates at the Holliday junction stage (3). These peptides stabilize the protein-bound form of the Holliday junction (4, 5). In the case of one of these peptides, WKHYNY, and its derivatives, the initial DNA cleavage event is not affected (5), whereas in the case of the peptide KWCRW and its derivatives, initial DNA cleavage is blocked at high concentrations of the peptide (4).

Phage λ integrase (Int),1 like many of the phage-encoded tyrosine recombinases used to establish lysogeny, performs site-specific recombination between different pairs of DNA substrates: between attP and attB during integration of the phage chromosome into the host’s chromosome and between attL and attR during excision (Table I) (2). In addition to these two pathways, Int catalyzes recombination between two attL partner sites; the IHF-stimulated bent-L pathway is as efficient as integration and excision, whereas the IHF-inhibited pathway is very inefficient (6, 7). When we tested the ability of the WKHYNY and KWCRW hexapeptides to trap Holliday junctions in these pathways, we found that they are most effective at trapping junctions in bent-L recombination and integration but 10–20-fold less effective in excisive recombination (4, 5). Peptides WKHYNY and KWCRW were isolated using the bent-L pathway during the screening process. In order to understand how the target of the peptides differs among the Int-mediated pathways, we repeated our screen for peptides using excisive recombination as the assay. We have identified a new set of hexapeptides that act as dimers and trap Holliday junctions in the excision pathway as well as in the other pathways. Like peptide KWCRW, they also inhibit DNA cleavage by type I topoisomerases and restriction endonucleases at high concentration. Reversal of the dimerization by DTT results in dissociation of the peptides from the Holliday junction complexes and allows their resolution by the stably bound Int. We now have a way both to stop the reaction in the middle and measure the accumulation rate of Holliday junctions and to release the block and measure the disappearance rate of junctions due to resolution in the context of the actual reaction intermediates.

**EXPERIMENTAL PROCEDURES**

DNA Substrates and Proteins—DNA substrates were generated by PCR using plasmid templates containing cloned attL, attLen1, attR, attB, or lax sites. Substrates were radiolabeled at the 5′-end with [γ-32P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (New England Biolabs). Supercoiled attP and pUC19 plasmids were isolated from *Escherichia coli* strains and purified using the Promega Wizard Plus SV Midiprep kit. Int was expressed under the control of a

*This study was supported by NIGMS, National Institutes of Health, Grant R01-52847 (to A. M. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Int, integrase; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; BMH, bismaleimidohexane; LJ, Holliday junction; HJC, Holliday junction complex; DTT, dithiothreitol; IHF, integration host factor.
Reversible Inhibitors of Holliday Junction Resolution

1. Catalytic events mediated by Int during site-specific recombination (adapted from Ref. 21). The Int protein together with accessory factors juxtaposes the two recombination substrates in a synaptic complex. The active site tyrosine of each Int monomer attacks a specific phosphodiester linkage and forms a transient covalent 3'-phosphotyrosyl bond between the enzyme and the top strand of each DNA substrate. Ligation occurs when the free 5'-OH group from a partner substrate (or from the original substrate) acts as a nucleophile at this phosphotyrosyl linkage. Since the two DNA strands of each substrate are cleaved independently, a Holliday junction is generated during recombination. The complex undergoes an isomerization, which positions the previously inactive Int monomers into the appropriate conformation for subsequent catalytic steps. The Holliday junction is resolved by a repetition of the previous DNA cleavage, strand exchange, and ligation steps on the bottom strand of each DNA substrate, resulting in two recombinant DNA molecules.

2. C. Robertson and H. A. Nash, personal communication.
tions tested were 100, 66, 33, and 1 μM. Reactions were incubated for 60 min at 37 °C, stopped with 0.25 volumes of 2% SDS containing xylene cyanol and analyzed as the human topoisomerase reactions.

E. coli Topo III assays were performed in a volume of 20 μl and contained 200 ng of supercoiled pUC19 plasmid DNA, 25 nM enzyme, 40 mM HEPES-KOH (pH 8), 1 mM magnesium acetate, 0.1 mg/ml bovine serum albumin, and 40% glycerol (15). Peptides were tested at a concentration of 50 μM. Reactions were incubated at 52 °C for 30 min, and gel loading dye (15% Ficoll, xylene cyanol, and bromphenol blue) (16) was added. Reactions were loaded on a 1% agarose gel and electrophoresed at 60 V, whereas subsequent steps were identical to analysis of human topoisomerase reactions.

Restriction Enzyme Assays—Reactions were performed in a volume of 20 μl and contained 1× appropriate New England Biolabs reaction buffer, 200 ng of pUC19 supercoiled plasmid DNA, and 10 units of NdeI or HindIII enzyme. Peptide concentrations tested were 100, 75, 50, 25, and 5 μM. Reactions were incubated for 30 min at 37 °C, allowing roughly 50% cleavage of the substrate. Reactions were then separated on agarose gels and visualized as described above for human topoisomerase.

Peptide—Peptide libraries were synthesized with a C-terminal amide group using TBOC-protected amino acid residues (17) at the Torrey Pines Institute for Molecular Studies. Peptides of specific sequence, also with a C-terminal amide, were synthesized at Sigma-Genosys using Fmoc (N-(9-fluorenylmethoxycarbonyl)-protected L- or D-amino acid residues. The dual defined position peptide libraries and the final peptides were solubilized in 50–100% Me2SO; therefore, we tested the effect of Me2SO on recombination in each pathway and found no effect at the concentrations used: at most 1% and typically much less than 1% (data not shown). WRWYCR and WRWYAR homodimers linked to each other via the tether separating the mono and dipeptide group using TBOC-protected amino acid residues (17) at the Torrey Pines Institute for Molecular Studies. Peptide structures and purity levels were verified by high pressure liquid chromatography and/or by mass spectrometry.

Bismaleimidohexane (BMH)—cross-linked Peptide—WRWYCR was cross-linked with BMH, an irreversible homobifunctional cross-linker reactive to sulfhydryl groups with a 16.1-μM concentration of 0.5 μM to trap the junction intermediates. Typically, 4–8 × 50-μl (2.5×) reactions were necessary to provide sufficient substrate for several experiments. The HJs were isolated by running the reactions on a Tris-SDS-5% polyacrylamide gel (29:1), exposing to film, and excising the appropriate band. The DNA was isolated from the gel using a wash/soak passive elution into Tris-EDETA (10 mM:1 mM) with 0.1% SDS followed by centrifugation through an empty spool column. The DNA was precipitated using ethanol and sodium acetate, resuspended in Tris-EDETA (10 mM:1 mM), and quantitated by A260. The purified HJs were added, at a final concentration of 2 nM, to a reaction identical to that in which they were generated, except that proteins were absent. This reaction is held at 37 °C and initiated by the addition of a mix consisting of (room temperature-pretreated) Int (50 nM final concentration), IHF (35 nM), and Xis (50 nM). After the indicated time, the reactions were stopped with SDS at a final concentration of 1% and analyzed as described above. Note that if the proteins are preincubated with the substrate at 4 °C, significant resolution (about 6–10% in 5 min) occurs.

The Holliday junctions for the “DTT release” assays were formed in normal recombination reactions containing linear attL and attR substrates, Int, IHF, and Xis and peptide WRWYCR at 0.5 μM, as above. After incubating for 45 min to allow the maximum HJs to form, 10 mM DTT (final concentration) was added. The reduced peptides dissociate, allowing the bound Int to resolve the junctions. The addition of DTT is considered t = 0 with respect to junction resolution, and the reactions are stopped at various times and analyzed as above.

RESULTS

Previously, our laboratory has identified several hexameric peptide inhibitors potent in the accumulation of Holliday junctions by screening synthetic peptide combinatorial libraries using a positional scanning strategy (3). The bent-L pathway was used as the target of the screen, an efficient Int-mediated pathway with relatively simple requirements (Table I) (7). Whereas the original peptides were quite potent in this pathway, their potency was as much as 10–50-fold lower in other Int-mediated pathways (Table II) (4, 5). We hypothesized that, by rescreening peptide libraries using the excision pathway, we may identify a more potent inhibitor of excision. This has indeed been the case.

Peptide Library Screening—To screen for excisive recombination inhibitors that lead to accumulation of pathway intermediates, we used the same positional scanning approach described previously for the identification of inhibitors of Int-mediated recombination (3, 17), illustrated in Fig. 2. The first step involved testing the effect on excision of 20 separate hexapeptide mixtures, each of which has one of the 20 amino acids fixed at one of the six positions and an equimolar mixture of 19 amino acids (all except cysteine) at the other five positions. Each mixture was tested at two concentrations at least twice; the concentrations of the mixtures were 3–10-fold lower than in our previous screen (3). In addition, each reaction contained 100 ng of salmon sperm DNA in order to minimize the effect of nonspecific DNA binding by the peptides. The individual peptide mixture was added to an excision reaction, incubated for 60 min, and analyzed on a Tris/Tricine/SDS gel. Holliday junctions accumulated in each reaction were quantitated, and the mixtures were ranked accordingly. This process was repeated with mixtures in which each of the other five positions was fixed in turn. A total of 120 peptide mixtures were tested during the first step (Fig. 2). The most active mixtures were compared with each other in more extensive dose-response assays in order to reduce the number of candidate residues at each position to four or five (residues marked by asterisks in Fig. 3A).

In the second screening step, we used dual defined mixtures in which pairs of positions in the hexapeptides were fixed with all combinations of the most effective residues identified in the first step of the deconvolution (Fig. 2). As before, the dual defined mixtures were significantly more inhibitory than the single defined mixtures (3, 17); the most potent single fixed
mixtures accumulated 20–40% junctions at 30 μg/ml final concentration (Fig. 3A), whereas many of the dual defined mixtures accumulated the same amount of junctions at 5 μg/ml final concentration (Fig. 3B). This is most likely due to the higher concentration of individual peptides in the dual defined mixtures. Four hundred mixtures represent all possible combinations of two amino acids (20 × 20) at one pair of neighboring positions, and 1200 mixtures represent the entire peptide (three nonoverlapping pairs of contiguous amino acids). By confining ourselves only to the combinations of the most effective amino acids identified in step 1, we reduced the number of mixtures we tested from 1200 to 108. The best candidate mixtures were again compared with each other in dose-response assays, and the most effective dual defined mixtures selected for inclusion in the final defined sequence peptides are marked in Fig. 3B. We found that, although Trp emerged as the most potent single residue at most positions in the mixtures with a single fixed position (Fig. 3A), mixtures having two adjacent Trp residues were never among the most potent dual defined mixtures (Fig. 3B).

In most cases, the amino acids identified by the two most effective dual defined mixtures were selected for inclusion in the final peptides. Eight peptide sequences were chosen based on ability to accumulate the most Holliday junctions; in this case, a single pair of amino acids was chosen for positions 1 and 2 (WR; the second best pair, RW, was very close in potency but closely resembled the KW sequence of previously isolated peptides (4)). Four candidate amino acid pairs were selected for positions 3 and 4, and two candidate pairs were selected for positions 5 and 6. The remaining six peptide sequences were chosen based on the ability to inhibit recombination without necessarily accumulating junctions at the highest level. In this case, four possible pairs of amino acids were considered at positions 1 and 2, whereas the remaining four positions were the same for all peptides, since they clearly “outperformed” mixtures with other defined amino acid pairs. In all, 14 hexapeptides were selected, and 13 of these were tested (Table II; the synthesis of one peptide, YWWWWW, failed). Although we designed the final peptides based on two different phenotypes, this distinction did not hold in the final analysis. If we had proceeded directly from step 1 to designing specific peptides based on all combinations of the top four residues identified at each position initially, we would have had to synthesize 4096 different peptides, a cost-prohibitive approach.

We identified several unique peptides proficient in accumulating Holliday junctions, the most potent of which were WRRWCR and WRWYCR. The effect of these peptides in the excision pathway is shown in Fig. 4. At a 0.5 μM concentration of each peptide, very few excision products form, coincident with the highest accumulation of Holliday junctions. This suggests that, at this concentration, formation of recombination products is prevented because the substrates are trapped at the Holliday junction stage. The concentration at which 50% of the maximal amount of HJJs are trapped is 0.02 μM, which coincides with the concentration that reduces the amount of recombination products by 50% (Fig. 4B). We defined this concentration as the IC_{50} for excisive recombination. At concentrations lower than 0.005 μM, the peptides are no longer effective at accumulating Holliday junctions above the background seen in untreated reactions. At 28 μM peptide, excision is completely inhibited with no accumulation of junctions (Fig. 4A) presumably because the initial DNA cleavage events are also blocked. This behavior is similar to that of peptide KWWCRW, which also inhibits DNA cleavage at high concentration (4). Because peptides WRRWCR and WRWYCR have very similar effects, in the future we will refer primarily to peptide WRWYCR, which is slightly more potent at inhibiting some pathways. The IC_{50}

### Table II

| Peptide | Ex | In | B-L | S-L | Cre | dsDNA |
|---------|----|----|-----|-----|-----|-------|
| WRYWYCR | 0.21 | 0.009 | 0.005 | 0.25 | 0.75 | Y     |
| WRRWCR  | 0.032 | 0.01 | 0.028 | 0.25 | 0.05 | Y     |
| WRWYCR  | 0.045 | NT   | 0.045 | NT   | NT   | Y     |
| WRRWYCRW | 0.075 | 0.005 | 0.045 | 2.0  | 0.3  | Y     |
| YWWYCRW | 0.11 | 0.045 | 0.018 | 0.55 | 0.2  | N     |
| RCYKYW   | 0.12 | 0.13 | 0.009 | 1.5  | 0.07 | Y     |
| WRYCRW   | 0.16 | 0.009 | 0.005 | 15.0 | 0.3  | Y     |
| WRYWYWW  | 0.18 | 0.25 | 0.04 | 10.8 | 0.2  | Y     |
| WRYWYCRW | 0.44 | 0.21 | 0.1  | 0.46 | 0.1  | Y     |
| WRWYWWW  | 0.65 | 10.0 | 0.065 | 15.0 | 0.3  | Y     |
| WYYYWWW  | 0.7 | 3.5 | 0.25 | 4.0  | 0.1  | N     |
| WRWYRW   | 0.6 | 0.61 | 1.5 | 2.25 | 2.0 | +/+    |
| KKWYRW   | 1.0 | 0.5 | 0.02 | 0.06 | NO | Y     |
| KKWYWRY  | 1.1 | 0.2 | 0.77 | 0.1  | QO | Y     |
| WRRWYRW  | 1.3 | 0.35 | 0.8 | 0.90 | 0.05 | +/-   |
| WRRWYRW  | 1.3 | 1.1 | 1.6 | 2.00 | 0.05 | +/-   |
| WRRWYCRW | 2.0 | 2.2 | 0.2 | 20.0 | NO | N     |

* Peptides are arranged in order of decreasing potency in the excision pathway; the lighter the shading, the higher the potency.
* Ex., excision; In., integration; B-L, bent-L recombination; S-L, straight-L recombination.
* dsDNA, double-stranded DNA binding; N, no binding to dsDNA; Y, significant binding to dsDNA (see Fig. 6).
* Small lettering denotes peptides synthesized from D-amino acid; NT, not tested; QO, not quantitated.
* Data for the peptide in italics was taken from Klemm et al. (4).
The value of these peptides is over 50-fold lower for excisive recombination than the IC_{50} values of the previously identified peptides, WKHYNY and KWWCRW (4, 5). Like these latter peptides, the newly isolated peptides did not cause the accumulation of covalent protein-DNA intermediates, which indicates that none of them affects the ligation step of strand exchange (3–5).

Peptides WKHYNY and KWWCRW were shown to stabilize protein-bound Holliday junctions rather than free junctions (4, 5). Therefore, we tested whether the newly isolated peptides also accumulate protein-bound Holliday junctions. Recombination proceeds by assembly of successive nucleoprotein complexes on each individual att site (unimolecular complexes or UMCs), followed by the formation of synaptic complexes (bimolecular complexes or BMCs) (9, 18, 19). Strand cleavage occurs either in the UMCs or BMCs, depending on the substrate (20).³ whereas strand exchange (formation and resolution of Holliday junctions) occurs within the BMCs. However, the excision BMCs are too unstable and can only be seen by using the previously identified peptides such as WKHYNY (5). We electrophoresed untreated and peptide-treated excision reactions on a native gel rather than on a protein-denaturing gel. In the presence of peptide, the amount of complex corresponding to the attP unimolecular product decreased, and a new, slower migrating complex appeared (Fig. 4C, left) that depended on the presence of both att sites, Int, IHF, and Xis (data not shown). A gel slice representing all of the products of a peptide-treated reaction was excised and electrophoresed on a protein-denaturing gel and was found to contain a mixture of Holliday junctions, unreacted substrate, and a small amount of recombinant products (Fig. 4C, right bottom panel); the new complex was named the excision Holliday junction complex (EX-HJC). A similar gel slice taken from an untreated excision reaction showed no Holliday junctions at all and only a small amount of complexes containing covalently bound Int molecules, the covalent protein-DNA intermediates (Fig. 4C, right bottom panel).

³ G. Cassell and A. Segall, unpublished results.

Fig. 3. Results of screening single and dual defined peptide mixtures. A, the effect of single defined peptide libraries on excisive recombination. Recombination reactions were treated with mixtures with a single position defined at the final concentration shown in the figure. The amino acid residue at the fixed position is denoted along the x axis. Values for percentage of HJs in the reaction were calculated as the radioactive counts measured for the junction expressed as a percentage of total counts in the lane. The mixtures chosen for the second step of deconvolution are marked with an asterisk. These mixtures were chosen not only on the basis of the data shown here but also based on the results of dose-response assays with the top six candidate mixtures at each position (the concentrations used for the dose-response assays were between 1 and 0.0003 mg/ml final concentration). On occasion, if two closely related residues (e.g. lysine and arginine) were among the top candidates at a position, only one of these was chosen for the second step to facilitate the inclusion of more unique residues. B, the effect of dual defined peptide libraries on excisive recombination. 8–16 mixtures were tested for each position pair. Shown is the activity of the most potent six mixtures. The final total concentration of peptides added to each reaction is shown. Values for percentage of HJ accumulation were calculated as in A. The minimum and maximum amounts of HJs accumulated from all of the dual defined mixtures tested for each library are indicated under the x axis. The mixtures that were most potent at blocking recombination after dose-response assays and that consequently were chosen for inclusion in final peptides are marked with an asterisk.
Mechanism of Inhibition

We next tested how peptide WRWYCR affected the formation of other nucleoprotein intermediates in the excision reaction. WRWYCR, like the previously identified peptide inhibitors (4, 5), does not impair formation of the UMCs; nor does it substantially increase the amount of free DNA in either the excision reaction (Fig. 4C, left panel) or the bent-L pathway (Fig. 5A, lane 2 versus lane 7). HJCs accumulate at about the same levels even in the presence of 1 μg of nonspecific DNA in the reaction (Fig. 5, A and B), suggesting that the peptides bind significantly better to HJCs than to double-stranded DNA. Indeed, we hoped to achieve this specificity by including nonspecific DNA during the screening and deconvolution.

In order to test directly the affinity of the newly identified peptides for double-stranded DNA, a mobility shift assay was performed with two different doses of each peptide and the attL substrate in the absence of any recombination proteins. The same amount of nonspecific DNA, 100 ng, was present as during the deconvolution steps. Based on their ability to shift
the att site into the well and to diminish the amount of DNA migrating as free probe, most of the peptides showed significant affinity for double-stranded DNA at 5 μM, and a few showed some double-stranded DNA binding activity even at 0.5 μM (Fig. 6). However, in the case of peptides WRRWCR and WRRWCR, the IC₅₀ for Int-mediated recombination is around 20 nM (the straight-L pathway excepted), 25-fold lower than 0.5 μM. Thus, it is very unlikely that inhibition is due to nonspecific interactions of the peptides with DNA. All together, these results indicate that the most potent peptides recognize a conformational interface specific to the Holliday junction recombination intermediate. Indeed, these peptides also bind to protein-free Holliday junctions.⁴

The stability of interactions between peptides WRRWCR, WRRWCR, and YWCVYWW and the IIJC was tested using a dilution assay. Excision reactions were incubated in a volume of 10 μl for 60 min in the presence or absence of peptides and then diluted 20-fold with reaction mix lacking peptide or additional recombination proteins and were incubated a further 10 min. In reactions initially assembled in a 200-μl reaction volume, fewer junctions accumulated when compared with reactions assembled in 10 μl (Fig. 7, peptide-treated reactions). If the peptides interacted with the HJC weakly, they would have dissociated when diluted, and the Holliday junction will have been resolved into recombination products. Peptide YWCVYWW demonstrated the most stable interactions and did not allow any recombinant products to form after dilution (data not shown). Peptide WRRWCR allowed 5% products to form, compared with 35% products formed in reactions assembled in 10 μl (Fig. 7 and data not shown). Of the three peptides tested, WRRWCR interacted most weakly with the junction complexes, allowing about 50% of the HJs to proceed to recombination products when diluted (Fig. 7).

Inhibition of Other Int- and of Cre-mediated Recombination Reactions—We next tested the potency of the newly identified peptides in the other three Int-mediated pathways, integration, bent-L, and straight-L. The IC₅₀ values for all peptides in each of these pathways is shown in Table II. Peptide WRYWCR has an IC₅₀ value of 0.005 μM in the bent-L pathway, 0.009 μM in the integration pathway, and 0.25 μM in the straight-L pathway, and peptide WRRWCR was nearly as potent. In general, the most potent peptides in the excision pathway were also the most potent in the other pathways.

In order to determine the specificity and mechanism of inhibition by the peptides, we tested their effect on reactions mediated by several other proteins, related or unrelated to Int. Cre, a site-specific recombinase encoded by phage P1, is mechanistically and structurally closely related to Int (1). Most of

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⁴ K. Kepple, J. L. Boldt, and A. Segall, submitted for publication.
the peptides we identified inhibit Cre-lox recombination and Int-att recombination with similar potency, with peptide WRWYCR the most potent (Table II). In contrast, peptide Int-RRR was not as potent in the Cre system, whereas peptide WRWYCR was the most potent (Table II). In contrast, peptide WRWYCR is a dimer assembled via a disulfide bridge between two peptide monomers, increasing the local concentration of peptide. To test whether the active form of peptide WRWYCR is indeed a dimer, two copies were linked together by synthesizing each monomer from the α and ε amino groups of Lys at the C terminus of the peptide. A similar construct was made with two copies of WRWYAR, and both are referred to as Lys-dimers. Bent-L recombination assays were performed with the modified peptides at different concentrations to determine the effect of the modification and of DTT addition on their potency. When comparing the resistance of these peptides with DTT, we tested three concentrations: 1) the IC_{50} concentration for the specific peptide; 2) the concentration where maximal accumulation of HJs was obtained; and 3) a concentration intermediate between the previous two.

The Lys-dimer forms of WRWYCR and WRWYAR trapped nearly the same fraction of substrates as HJs (a difference of only 12%) both in the presence and in the absence of DTT at all concentrations tested (Fig. 8B). (Note that at the higher concentrations these peptides, like the monomeric versions, inhibit DNA cleavage and therefore have fewer HJ substrates to trap.) In contrast, the hexapeptide WRWYAR is completely inactive tested (data not shown). Finally, the HindIII and NdeI restriction enzymes were selected as DNA-transacting enzymes that have no mechanistic or structural similarity to Int. The cleavage activity of both was inhibited to the same extent as the type IA and type IB topoisomerases, probably due to the peptides’ nonspecific double-stranded DNA binding activity.

**Contributions of Length and Sequence to Peptide Function**—We investigated the minimal length required for inhibitory activity of peptides WRWYCR and WRWYCR. Pentapeptides were about 10-fold less potent than the full-length peptide and were unable to accumulate HJs to the same extent as the full-length peptide (Table III). When comparing the potency of pentapeptides versus that of hexapeptides, sequence is at least as important as length. Removal of the Cys residue is very deleterious. In addition, at least one Arg is necessary for maximal peptide function, and its position within the peptide is important. None of the tetrapeptides we have tested show significant activity in our assays when compared with the pentapeptides, even when they contain the residues most important for activity of the parent peptide. Thus, the length of the peptides, presumably due to spacing or fit into the binding pocket, is very important for the inhibitory activity.

We investigated the contribution of each residue in peptide WRWYCR to its function by replacing each of its residues individually with alanine. The substitution of residues at position 1, 2, 4, or 6 with alanines affected the inhibitory phenotype only modestly in excision (Fig. 8A). Substitution of tryptophan with alanine at position 3 had a larger impact, suggesting that this residue is important for the potency of the peptide. The most drastic loss of activity occurred when the cysteine at position 5 was replaced with an alanine, reducing the activity of the peptide to 4%. This phenotype suggested that a disulfide bridge between two peptide monomers may play a critical role in the mechanism of inhibition.

**The Active Inhibitor Is a Disulfide-bridged Hexapeptide Dimer**—The addition of DTT greatly reduces the activity of peptide WRWYCR. At the IC_{50} concentration of peptide WRWYCR, the amount of junctions trapped was reduced by 82% in the presence of 50 μM DTT. As more peptide was added, the amount of trapped HJs in the presence of DTT increased such that at 5 μM peptide, DTT had no effect on inhibition of recombination or junction accumulation (Fig. 8A and data not shown). This supports our hypothesis that the active form of peptide WRWYCR is a dimer assembled via a disulfide bridge between two peptide monomers, increasing the local concentration of peptide.

**Inhibition of Other DNA-transacting Enzymes**—To determine the specificity of each of the peptides identified in this screen, they were tested in a dose-response assay against several enzymes related and unrelated to Int (23–28). The peptides exhibited dose-dependent inhibition of plasmid relaxation by vaccinia virus topoisomerase and by human topoisomerase I, both type IB topoisomerases. However, the peptides were 400–20,000-fold less potent against the type IB topoisomerases than they were against Int (data not shown). The peptides inhibited plasmid relaxation by the *E. coli* topoisomerases I and III, which are type IA topoisomerases, even less well than they inhibited relaxation by the type IB enzymes; often, no effect was seen at 100 μM peptide, the highest concentration
Reversible Inhibitors of Holliday Junction Resolution

Structure/function analysis: Effect of amino acid substitutions or deletions

| Peptide     | IC_{50} (without DTT) | IC_{max} (without DTT) | Max HJ, % | Relative activity |
|-------------|-----------------------|------------------------|-----------|------------------|
|             | µM                    | µM                     |           |                  |
|             |                       |                        | Without DTT | With DTT         |
| WRWYCR      | 0.018                 | 0.5                    | 100        | 34 (+)           |
| WRWC       | 0.07                  | 0.5                    | 61         | 9 (5) (+)        |
| WRWC       | 0.11                  | 5                      | 77         | 11 (+)           |
| WKWYC      | 0.12                  | 5                      | 55         | 2 (+)            |
| WRWC       | NA                    | 50                     | 14         | <1 (5) (+)       |
| WRWC       | NA                    | 50                     | 25         | 25 (+)           |
| WRRWC      | NA                    | 50                     | 31         | 38 (+)           |
| WYCR       | NA                    | 50                     | 29         | 9 (+)            |
| WYCR       | NA                    | 50                     | 16         | 45 (+)           |
| WYCR       | NA                    | 50                     | 23         | 16 (+)           |
| WRRWCR     | 0.04                  | 0.8                    | 100        | 13 (0.5) (+)     |
| WRWC       | 0.2                   | 5                      | 56         | 4 (+)            |
| WRWC       | 0.35                  | 5                      | 47         | 2 (+)            |
| WRRWC      | NA                    | 5                      | 80         | 107 (+)          |
| WYCR       | 0.2                   | 5                      | 87         | 2 (+)            |
| WYCR       | NA                    | 50                     | 5          | <1 (5) (-)       |

1 IC_{max}, concentration at which maximum amount of junctions was observed
2 Max HJ, maximum percentage of junctions observed at the IC_{max} concentration given. The amount of HJs accumulated by peptides WRRWCR and WRWYCR is defined as 100%; the amounts of HJs accumulated by the other derived peptides are given with respect to the “parent” hexamer, in boldface type. All peptides were tested at 0.005, 0.05, 0.5, 5, and 50 µM.
3 NA, maximum percentage of junctions observed either at the IC_{max} concentration or at the concentration given in parentheses.

Reversibility of Inhibitors

The following reagents were used to reconvert the complexes to their original state: captoethanol, (without DTT) K. Kepple and A. Segall, unpublished results.

in bent-L recombination and only weakly active in excision at 0.5 µM; its IC_{50} is shifted with respect to WRWYCR such that it traps the maximum Holliday junctions at the highest concentration tested, 50 µM, in both pathways (data not shown). Dimerized WRWYCR had a 10-fold decrease in potency of inhibiting recombination, whereas dimerized WRWYCR had a 50-fold decrease in potency in the absence of DTT (Fig. 8B). The lower potency of these peptides may be due to the reduced mobility imposed by the Lys tether and/or the fact that the Lys tether displays the two peptide monomers asymmetrically with respect to each other. This could make it more difficult for these Lys-dimer peptides to associate with the IHJ intermediate. Another possibility is that the Lys-dimers interact more stably with nonspecific DNA compared with the monomer peptides, which reduces their effective concentration.6 These peptides do shift Holliday junction DNA, just like the monomeric versions, but the resulting complexes are DTT-resistant.5

We also dimerized two copies of WRWYCR by cross-linking them with BMH, a homobifunctional cross-linking agent reactive with sulfhydryl groups. Although this modification slightly decreased the amount of HJs trapped by the peptide in the absence of DTT, it is almost completely resistant to 50 mM DTT (Fig. 8B). Taken together with the activity of the Lys-dimers, this result provides strong evidence that the active form of peptides WRWYCR and WRRWCR is a homodimer.

The sensitivity of the pentat- and tetrapeptides to DTT was also tested. Because the pentapeptides and especially the tetrapeptides are less potent than the hexapeptides, in these assays we added enough peptide to see some inhibition of recombination in the absence of DTT (the concentration used varied with peptide; see Table III). We then added DTT to test the importance of the reduced versus the oxidized state of the peptides. Most derivatives of peptide WRRWCR were highly sensitive to DTT, losing their ability to accumulate HJs (Table III). The pentapeptide derivatives of WRWYCR were also sensitive to DTT. In contrast, many of the tetrapeptides were not sensitive to DTT, and one of them, WYCR, was stimulated 3-fold in the presence of DTT, suggesting that a disulfide bridge in fact hinders the binding and/or activity of these tetrapeptides. One interesting comparison is between the l-WWCR and d-wwr peptides; the former is slightly stimulated, whereas the latter is severely inhibited by DTT. We do not yet understand the basis for this difference.

Holliday Junction Resolution—To date, most studies of Holliday junction resolution have been performed by assembling artificial Holliday junctions in vitro from constituent single strands and “reloading” these junctions with junction-resolving enzymes (29–31). First, we do not know how closely these reloaded junction complexes resemble the complexes generated during the normal course of recombination. Second, fewer than 7% of reloaded junction complexes in the bent-L pathway of Int-mediated recombination are resolved (5), despite the fact that this pathway is as efficient as excisive or integrative recombination (summarized in Table 1) (7, 9). Therefore, it has been impossible to determine the bent-L rate of resolution or to investigate the Int residues that affect junction resolution in this pathway (20). The new inhibitory peptide WRWYCR provides a solution to these problems. As shown above, the active form of the WRWYCR peptide is a dimer linked by a disulfide bridge, which, when reduced by the addition of DTT or β-mercaptoethanol, generates peptide monomers that do not effectively trap the Holliday junctions. Based on this property, we have designed a new assay for Holliday junction resolution, which we have named the “DTT release” assay. In this assay, recombination reactions are assembled and incubated with peptide for 45 min (1st Incubation), to allow most of the substrates to proceed to the Holliday junction stage (Fig. 9, lane 3 versus lane 6; also see Refs. 3 and 5). At 45 min, 50 mM DTT is added in order to reduce the disulfide bridge and inactivate the peptide. DTT added to the recombination reaction in the absence of peptide is not detrimental and in fact slightly increases recombination efficiency (Fig. 9, lane 2 versus lane 4). A “second” incubation is carried out for various lengths of time, permitting the proteins in the complex to resolve the junction. As shown in lanes 7–12 in Fig. 9, the longer the second incubation (2nd Incubation), the greater the accumulation of resolved products, until almost all of the junctions are resolved to products (Fig. 9, lane 12 versus lanes 5 and 2). We then calculate the rate of disappearance of the Holliday junctions (the resolution rate). Holliday junction resolution rates were measured in par-
For the activity of the hexapeptide. All peptides were tested at 0.5 μM concentration in the presence or absence of 50 mM DTT, and the accumulation of HJs was measured. The concentrations were calculated based on the molecular weight of the monomeric peptide to allow direct comparison with the unmodified form.

We found that in excisive recombination, the rate of reloaded junction resolution is roughly the same as the rate of DTT-released junction resolution (Table IV). Each of these assays has features not present in the other. In the case of reloaded junctions, the proteins have to associate with the junction DNA before catalyzing resolution, whereas in the DTT release assay the proteins are already present in the complex. The reloaded junction assays were performed in two ways; either proteins were first incubated with the junction substrates on ice to allow binding and then shifted to 37 °C to allow efficient catalysis.

The rate in parentheses was obtained in experiments in which the proteins were first incubated with the junction substrates on ice to allow binding and then shifted to 37 °C to allow efficient catalysis. *NA, not applicable.

**Fig. 9. DTT release assay: Resolution of trapped HJs by DTT addition.** All reactions contain labeled attL substrate and unlabeled partner attR as well as 100 ng of nsDNA. **Lane 1,** negative control (absence of proteins). **Lane 2,** positive control; recombination after 75 min in the absence of peptide. **Lane 3,** the amount of HJs trapped with peptide added at a final concentration of 0.5 μM, which generates the highest percentage of HJs. **Lane 4** shows that 50 mM DTT by itself has little effect on recombination (in fact is slightly stimulatory) but reverses the HJ trapping effect of peptide (lane 5). Lanes 6–12 show the extent of resolution of HJs trapped with peptide upon the addition of DTT as a function of time.

**Table IV**

| Treatment               | Reloaded junctions | DTT-released junctions |
|-------------------------|--------------------|------------------------|
| Excision                | 0.2 (0.14)         | 0.17                   |
| Bent-L                  | NA                 | 0.18                   |

All rates are expressed as fmol of the Holliday junction substrate converted to resolution products/μl pmol of Int. Initial rates were determined based on time points in which fewer than 20% of the Holliday junctions were resolved.

The rate in parentheses was obtained in experiments in which the proteins were first incubated with the junction substrates on ice to allow binding and then shifted to 37 °C to allow efficient catalysis.

| Lane | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 |
|------|----|----|----|----|----|----|----|----|----|----|----|----|
| **Proteins** | WRWYCR (0.5 μM) | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 1st Incubation | 75 | 75 | 75 | 75 | 45 | 45 | 45 | 45 | 45 | 45 | 45 | 45 |
| 2nd Incubation | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| DTT [50 mM] at t=0 | -  | -  | 0  | 0  | 45 | 45 | 45 | 45 | 45 | 45 | 45 | 45 |
| 2nd Incubation | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |

Fig. 8. **Structure/function analysis of peptides.** A, alanine scanning of peptide WRWYCR to test the importance of individual residues for the activity of the hexapeptide. All peptides were tested at 0.5 μM final concentration. B, effect of DTT on WRWYCR and several related dimerized peptides in the bent-L pathway. Each peptide was tested at the IC₅₀ concentration, at the concentration at which the maximal percentage of HJs accumulate, and at 10–20 times the previous concentration. The middle two peptides consist of two monomers that have been dimerized via linkage to the α and ε primary amines of a lysine residue. The last peptide consists of two WRWYCR monomers dimerized via BMH, an irreversible thiol-reactive homobifunctional cross-linker. Each of the peptides were added to reactions at the indicated concentration in the presence or absence of 50 mM DTT, and the accumulation of HJs was measured. The concentrations were calculated based on time points in which fewer than 20% of the HJs were resolved. Conversely, in the case of the DTT release assay, the extent of resolution of HJs trapped with peptide upon the addition of DTT is roughly the same as the rate of DTT-released junction resolution (Table IV). Each of these assays were performed in two ways; either proteins were added to the chilled reaction mixture, given 5–10 min on ice to bind, and then switched to 37 °C to allow efficient catalysis (“reloaded” chilled) in Table IV), or proteins were added to a prewarmed reaction mixture, and incubation was immediately started. The resolution rate observed in the case of the chilled reaction was about 25% slower than in the case of the prewarmed reaction (Table IV), presumably because of the length of time to warm the reaction mixture to 37 °C; therefore, we used the rate from the prewarmed reaction for our comparison. Conversely, in the case of the DTT release assay, the dissociation rate of the monomeric peptides and the time necessary for the complex to return to a catalytically competent state are part of the resolution rate. We have not yet measured directly the dissociation rate of the peptides due to technical challenges. However, the difference in resolution rates in the two assays is only ~12–13%, which could be the error margin of the experiment. Therefore, we think that it is reasonable to assume that the dissociation rate of the peptides and the time necessary for the conformation change to allow the junction complex to undergo catalysis are much faster than the catalytic rate. If not, this difference in rate may be due to a combination of all of the factors mentioned. Based on these data, it appears that the resolution rate of reloaded junctions provides an accurate measure of the junction resolution during recombination. Note that these rates are about 4-fold slower than the rate determined by Bankhead et al. (20), a difference due to slight differences in the exact conditions of the assays. The overall rate of recombination determined with the current conditions was 0.015 fmol of substrate converted to product/μl pmol of Int/s.

We next performed the DTT release assay using the bent-L recombination pathway. As mentioned above, reloaded junctions in this pathway show only ~5–7% resolution compared with nearly 100% resolution in the case of excisive reloaded junctions (5). We determined the rate of resolution using the DTT release assay and found that bent-L Holliday junc-
Inhibitors of biochemical pathways have been invaluable in studying a large spectrum of biological reactions, both in vitro and in vivo. The Holliday junction-stabilizing peptides we isolated previously have allowed us to measure rates of strand exchange and synapsis in both excisive and recombinative combination (20), which was not possible earlier. With the help of the peptides, we have been able to perform more thorough structure/function analyses of Int mutant proteins and of Int-catalyzed reactions (5, 20, 32). However, the original inhibitors, WKHYNY and KWWCRC, discriminated at least 10-fold between the different Int-mediated reactions and are most potent against the bent-L pathway and least potent against the excision pathway (4, 5). This raised the possibility that the Holliday junction intermediates in the different recombination pathways have distinct conformations and present different targets to the peptides. We have repeated the screen for inhibitory peptides using the excision pathway, and identified 13 new inhibitory peptides. These peptides vary in their ability to inhibit various reactions, with peptides WRWYCR and WRRWCR the two most potent. These new peptides also stabilize the protein-bound Holliday junction complexes. Like the previously identified peptide KWWCRC, at high concentrations they also inhibit catalysis by tyrosine recombinases, the mechanistically related eukaryotic type IB topoisomerases, and, to a lesser extent, the unrelated type IA topoisomerases and restriction enzymes. The latter activities correlate with nonspecific binding of the peptides to double-stranded DNA at high concentrations.

The most potent of the new peptides are significantly more potent than the previously isolated peptides, not just in excisive recombination, but in all Int-mediated pathways. Nevertheless, our initial assumption that different peptide sequences interact better with the intermediates of some pathways than others has been confirmed (Table II). Most of the peptides are more potent at trapping Int-generated junctions (the straight-L pathway excepted; see below) and less potent at trapping Cre-generated junctions. They are much less potent, as much as 400-fold less, at inhibiting the plasmid relaxation activity of type I topoisomerases or the DNA cleavage activity of restriction enzymes. Despite very closely related catalytic mechanisms and active sites, tyrosine recombinases generate Holliday junctions in the course of a reaction, whereas type IB topoisomerases do not. Thus, the higher inhibitory activity against the tyrosine recombinases supports our model that Holliday junction intermediates rather than double-stranded DNA are the target of the peptides. Our data can be accommodated by two models; either the peptides contact only the junction DNA itself, or they make contacts to both the DNA and protein residues. Either model can explain differences between the pathways and between the Int- versus the Cre-generated intermediates; in the former model, the peptides distinguish between the different junctions because the latter assume distinct conformations in the different Int pathways and in the Int versus Cre-mediated reactions. In the latter model, the differences in potency are due to the fact that the peptides cannot establish optimal contacts with the protein(s) in all of the Int-pathways or in the Cre reactions, but their contacts to DNA are sufficient. Indeed, the peptides specifically bind to protein-free Holliday junctions with a 400–1000 x preference over double-stranded DNA. However, this observation alone does not prove that the peptides contact only the DNA. More support for the DNA-only model is provided by the fact that the tyrosine recombinases share relatively little amino acid homology outside their active sites, yet we know that the peptides can accumulate junctions in Int-, Cre-, and Flp-mediated reactions; the last protein is particularly distant from the phage- and bacteria-encoded tyrosine recombinases (33, 34). At a minimum, we can deduce that protein contacts are not absolutely necessary for inhibition by the peptides.

Among the Int-mediated pathways, the bent-L reaction is most sensitive to inhibition, followed by integration and excision (note the color coding in Table II; the darker the shading, the less effective the inhibition). This is the case despite the fact that the peptides described here were identified as inhibitors of the excision pathway. In contrast, the straight-L pathway is less sensitive to inhibition by most of the peptides relative to the other pathways. In one way, this is counterintuitive; since our evidence suggests that the peptides inhibit recombination by blocking the resolution of the Holliday junction intermediates, a pathway that produces fewer of these intermediates should be more sensitive. Perhaps the conformation of the straight-L Holliday junction intermediates differs more from those of the other, efficient Int recombination pathways than the latter intermediates differ among each other.

The potency rank of peptides synthesized based on the deconvolution results together with the potency of peptides derived in structure from the most potent peptides has given us hints into the structure/activity relationships of these inhibitors. Peptides with WR in positions 1 and 2, and CR in positions 5 and 6 have the most inhibitory activity. In contrast, peptides with WR in positions 1 and 2 accompanied by RW in positions 5 and 6 are least inhibitory, unless RC are present in positions 3 and 4. Interestingly, whereas all of the most potent single fixed mixtures had Trp at the fixed position, peptide WCW-WWW performed comparatively poorly (Table II). This highlights that the results of the second, deconvolution step, in which combinations of amino acids are tested, is very important for selecting the most effective pairs of residues in the final peptides. Whereas the same conclusion could be reached based on results from the first, screening step, it would require synthesis of many more specific peptides and thus would be much more costly and time-consuming.

In general, the peptides that contain a cysteine residue are more potent than those that do not. Indeed, the oxidation state of peptides WRWYCR are WRRWCR is extremely important to their inhibitory potency; both the addition of reducing agents and the replacement of cysteine with alanine greatly reduce the activity of the peptides. Whereas the addition of DTT may counteract inhibitory activity in a variety of ways, the fact that dimerizing the peptides, either by linking them during synthesis through a lysine residue or by cross-linking them postsynthetically, strongly suggests that the active inhibitory form is a peptide dimer. Although these alternative dimerized peptides have 10–50-fold higher IC50 values, they accumulate Holliday junctions in the same way as the unmodified peptides; bind to Holliday junctions, and retain specificity. Because the Holliday junction intermediates of both Cre- and the Flp-mediated recombination are two-dimensionally symmetric (reviewed in Ref. 1), we surmise that the disulfide-linked hexapeptides are

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6T. M. Bankhead, E. Lipscomb, and A. Segall, manuscript in preparation.
arranged in antiparallel fashion in the central opening of the junctions. Testing the effect of peptide length on activity showed that some of the pentapeptides retain significant activity (within 10-fold of the hexapeptides), whereas the tetrapeptide derivatives have about 100-fold lower activity. All of these structure/function activity relationships provide a good start toward understanding the mechanism of action of the most active peptides. Based on this and structure/function information from our previously identified peptides, we also tested macrocyclic peptides with similar structures that we correctly predicted to inhibit Holliday junction resolution (35). However, the macrocyclic peptides were generally less effective at accumulating Holliday junctions in vitro than either our currently or previously identified linear peptides (4) (this work). Whereas it is too early to identify why, the higher flexibility of the linear peptides may be important for their association with the junction target.

How these peptides inhibit the resolution of junctions may best be characterized using x-ray crystallography of the tertiary enzyme-Holliday junction-peptide complexes. Indeed, the recombination intermediate between the wild type Cre protein and wild type lox substrates has been successfully crystallized in the presence of peptide WKHYNY, which prevented further catalysis.7 Previously, only catalytically defective Cre proteins and/or suicide substrates that prevented catalysis supported crystal formation (21, 36, 37). Indeed, the peptides also appear to promote crystal formation in the Flp-frt case.8

Peptides that accumulate Holliday junctions are proving to be extremely valuable tools for dissecting recombination pathways. The reversible inhibitors described here have allowed us to determine the resolution rate of junction intermediates in cases such as the bent-L pathway, where junction complexes reloaded with recombination proteins are not resolved (5). Our finding that the rates of Holliday junction resolution in the excise and bent-L pathways are the same when using the DTT release assay suggests that the conformation of junction complexes in the two pathways is quite similar with respect to protein-protein and protein-DNA interactions involved in HJ isomerization and resolution. However, this conclusion could be invalid if the dissociation rates of the peptide from the two complexes were different and perhaps rate-limiting. The latter is not the case: when we compared the rate of resolution of reloaded excision junctions with the rate of resolution of DTT-released excision junctions, the two rates were very nearly the same (Table IV), with the former (0.2 fmol/s-µmolInt) slightly faster than the latter (0.17 fmol/s-µmolInt). If this difference in rates is significant, it shows that the dissociation rate of the peptide from the complex contributes at most 15% to the resolution rate of the DTT-released junctions. However, this difference is within the error margin of our experimental methods, and it is unlikely that the resolution of Holliday junctions in the two pathways differ significantly. Nevertheless, reloaded bent-L Holliday junction complexes are very poorly competent for resolution (5). We have suggested previously that this may be due to the absence from the complex of a conformational feature that must be established early during the catalytic cycle. Thus, in the case of bent-L intermediates, the reloaded junctions are not a good model for junction resolution, and the DTT release assay provides a necessary alternative. Using the peptides, we are characterizing the fine structure of these intermediates and the nature of Int-Holliday junction interactions at this stage of recombination.

The peptides permit the detailed dissection of recombination pathways (e.g. Ref. 20). The combinatorial chemistry approach, and the use of mixture-based libraries in particular, allows the screening of very large chemical diversity in a relatively small number of mixtures for the identification of specific pathway inhibitors. This approach should be very broadly applicable to isolating stage-specific inhibitors of any biochemical pathway.

Acknowledgments—We thank Drs. Kaushik Ghosh, Gregory Van Duyne, and Phoebe Rice for sharing results prior to publication.

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Reversible Inhibitors of λ Integrase-mediated Recombination Efficiently Trap Holliday Junction Intermediates and Form the Basis of a Novel Assay for Junction Resolution

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J. Biol. Chem. 2004, 279:3472-3483.
doi: 10.1074/jbc.M309361200 originally published online November 18, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M309361200

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