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Analysis of a Nucleoprotein Complex: The Synaptosome of γδ Resolvase

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The γδ resolvase protein is one of a large family of transposon-encoded site-specific recombinases. It performs recombination in a DNA-protein complex that contains 12 resolvase protomers and two copies of the 120–base pair DNA substrate, *res* (each with three binding sites for a resolvase dimer). A derivative of resolvase with altered DNA binding specificity was used to show that the role of resolvase at site I, which contains the crossover point, differs from its role at the other two binding sites. The resolvase dimers that initially bind to site I are the only ones that require the residue Ser^{10}, essential for catalysis of DNA breakage. In addition, these site I–bound dimers do not use a specific interaction between dimers that is required elsewhere in the complex for synapsis of the *res* sites.

Large protein-DNA assemblies participate in DNA transactions such as transposition, site-specific recombination, and the initiation of replication (*1*). The DNA substrates often contain multiple binding sites for a protein that may function both as a structural component of the complex and as an enzymatic component, depending on its location in the complex. Examples of such assemblies include the transpososomes of phage mu and Tn7 (*2*), the intasome of phage λ (*3*), and the synaptosome of the resolvase family of site-specific recombinases (*4, 5*).

The γδ resolvase protein (encoded by the γδ transposon) carries out strand exchange within a synaptic complex that contains two 120-bp segments of DNA (known as *res*) and at least six dimers of resolvase (one dimer occupying each of the three binding sites that constitute *res*) (*4, 5*) (Fig. 1). The three binding sites within *res* are essential for its activity, and recombination occurs by DNA breakage and reunion at the center of site I. This synaptosome is a highly ordered structure—the two *res* sites are interwrapped, trapping precisely three negative superhelical turns (*6*)—and must be determined by a set of resolvase-DNA and resolvase-resolvase interactions constrained by the DNA linkage of the binding sites and by the negative superhelicity of the substrate. However, not all resolvase protomers in the complex are equal: only a subset provides catalytic function [defined here in the narrowest sense as providing the essential Ser^{10} residue that becomes covalently linked to the DNA during crossover site cleavage (*4*)], and it is likely that not all exhibit the same set of protein-protein interactions.

Our strategy for dissecting the structure and function of the synaptosome is to place specific mutants of resolvase—for example, those defective in chemical catalysis or in a defined set of protein-protein interactions—at specific binding sites within each *res* segment. The outcome of a resolution assay then indicates whether the resolvase bound at a defined site makes use of a specific protein-protein interaction during assembly of the synaptic complex or plays an active role in the chemical step of strand breakage. Specific binding to a mutant derivative of site I can be achieved with the resolvase mutant, R172L (with Arg^{172} replaced by Leu). This mutant was obtained by the use of a genetic screen (*7*) to select mutants of resolvase that could bind to a synthetic site I with a symmetrical G to T change at position 2 of the consensus half-site binding sequence TGTCCGATAATT (*8*).

We have used R172L to determine the location of the resolvase units that provide the active sites for catalysis of DNA breakage and reunion. The results of in vitro resolution reactions with pNG345 (a substrate containing the mutant site I^{G2T}) are shown in Fig. 2A (top panel). Two mutant resolvases were used: S10L, which lacks the critical Ser^{10} residue and binds weakly to site I (*9*), and R172L. S10L alone was

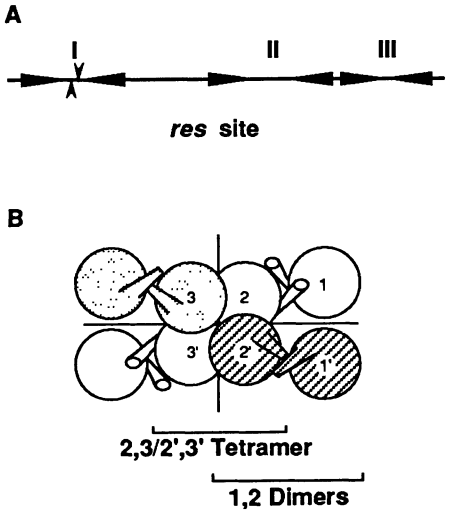

Fig. 1. Components of the synaptic complex assembled by γδ resolvase. (**A**) The DNA substrate, *res* (~120 bp) contains three resolvase binding sites, indicated by the inverted pairs of arrowheads and labeled I, II, and III. The vertical arrowheads in the center of site I indicate the recombinational crossover point. (**B**) An assembly of eight resolvase catalytic domains showing the various inter-resolvase interactions seen in resolvase crystals (*11, 17*). Four 1,2 dimers are shown (two shaded, two white) with the crossed cylindrical projections representing the pair of COOH-terminal α helices that interact at the 1,2 interface. (The un-numbered domains form 1,2 dimers with those numbered 3 and 3'). Each dimer contributes one monomeric unit to the 2,3/2',3' tetramer at the center of the assembly. The COOH-terminal DNA binding domains are not shown (they are not visible in resolvase crystals) but would be attached to the ends of the COOH-terminal α-helical projections (*17*). The lines indicate two of the three orthogonal dyad axes.

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completely inactive, whereas R172L alone showed barely detectable activity (presumably resulting from very weak binding to sites II^+ and III^+, which may be enhanced by cooperative interactions between resolvase dimers). In contrast, a mixture of the two mutants catalyzed efficient recombination of pNG345. This complementation was dependent on the specificity of the catalytically competent R172L mutant for site I^{G2T}, since no activity was seen with the substrate, pNG210 (with wild-type site I), except at the highest concentration of R172L, when in the presence of S10L a very low yield of products was observed (Fig. 2A, bottom panel). DNase I footprinting of the resolvases bound to *res*($I^{G2T}II^+III^+$) showed that the mutant proteins bound selectively and that full occupancy of the *res* site was obtained only when both mutants were provided (Fig. 2B). We conclude that the Ser^{10} component of the active site is provided by the resolvase protomers that specifically bind (by their COOH-terminal domain) to site I (*10*).

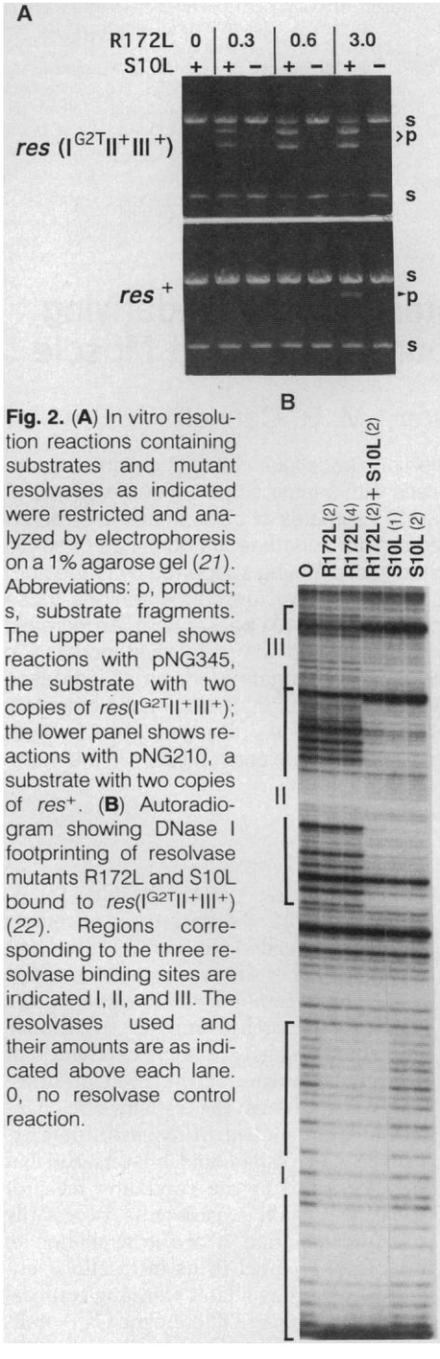

Fig. 2. (**A**) In vitro resolution reactions containing substrates and mutant resolvases as indicated were restricted and analyzed by electrophoresis on a 1% agarose gel (*21*). Abbreviations: p, product; s, substrate fragments. The upper panel shows reactions with pNG345, the substrate with two copies of *res*($I^{G2T}II^+III^+$); the lower panel shows reactions with pNG210, a substrate with two copies of *res*$^+$. (**B**) Autoradiogram showing DNase I footprinting of resolvase mutants R172L and S10L bound to *res*($I^{G2T}II^+III^+$) (*22*). Regions corresponding to the three resolvase binding sites are indicated I, II, and III. The resolvases used and their amounts are as indicated above each lane. 0, no resolvase control reaction.

The finding that the site I–bound resolvase performs the DNA breakage and reunion has important implications for the organization of the synaptic complex. The arrangement of resolvase protomers in crystals of resolvase or its NH_2-terminal domain led us to consider two alternative dimeric pairings for DNA binding and catalysis of recombination; these were called the 1,2 dimer and the 2,3 dimer (*11, 12*) (Fig. 1B). Recently, we have shown that the 1,2 dimer is the form of resolvase that binds to each subsite within *res* (*13*). However, certain features of the 1,2 dimer were unattractive for catalysis: the distance between the two Ser^{10} residues was not readily compatible with the DNA cleavage sites, and the dimer was not part of a 222-symmetric tetramer. In contrast, and for the opposite reasons, the 2,3 dimer appeared attractive for catalysis (*11, 12*). This has led us to consider two alternative hypotheses (*13*): (i) the 1,2 dimer also performs catalysis, but when bound to site I undergoes a conformational adjustment that brings the two Ser^{10} residues close to the DNA cleavage sites; (ii) catalysis is performed by a 2,3 dimer (in the context of the 222-symmetric 2,3/2′,3′ tetramer), with this catalytic unit formed specifically within the synaptic complex either from the 1,2 dimer that initially was bound to site I or from the assembly of resolvase protomers bound at the accessory sites II and III (and then recruited to the crossover point within site I). The results with R172L and S10L establish that the catalytic unit is not recruited from sites II or III and permit us to investigate the relevance of the 2,3/2′,3′ tetramer to the catalytic (site I–bound) unit of resolvase.

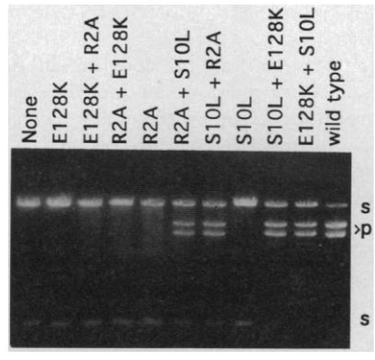

Fig. 3. In vitro resolution reactions containing the *res*$^+$ substrate, pRR51 (*23*), and mutant resolvases as indicated (*24*). Abbreviations are as in Fig. 2A. The faint band just visible below the resolution products is the result of cleavage at the crossover site without ligation.

The 2-3′ interaction, the core interaction of the 2,3/2′,3′ tetramer, is essential for synapsis and recombination and is eliminated in mutants such as R2A (*12, 14*). In principle, we could direct R2A to site I^{G2T} by constructing a double mutant with R172L. However, in vitro complementation experiments with a *res*$^+$ substrate and existing single mutants show that the R2A mutant is catalytically competent (Fig. 3). In pairwise complementation tests (Fig. 3), S10L and E128K (which cannot bind to site III) complemented each other [as shown before (*15*)], since the catalytically incompetent mutant, S10L, can bind at site III of *res* and perform the accessory functions needed at that site. By contrast, R2A did not complement E128K—even though R2A can bind to site III, it cannot take part in a 2-3′ interaction that presumably is required of the resolvase at site III for synapsis. However, a mixture of R2A and S10L showed substantial recombination activity. Our interpretation is that the catalytically incompetent S10L occupies sites II and III of each *res* segment and thereby effects synapsis, whereas R2A (assisted by the site I–binding defect of S10L) occupies both copies of site I and is competent to effect the pairing of these two sites as well as the breakage, exchange, and rejoining of the DNA at the crossover point. Since the R2A mutant is the only one (of R2A and S10L) with the active site Ser^{10}, and we have shown that the only binding site at which this Ser^{10} is required is site I, then R2A must be bound at site I to effect resolution. Thus, the data suggest that the unit of resolvase that binds site I and performs catalysis does not make use of the 2-3′ interface. We can draw two separate inferences (one strong, one weaker) from these data, since in the synaptic complex, the resolvase catalytic units at each site I are likely to interact both with one another (to pair the two crossover points) and also with resolvase dimers bound at the accessory sites II and III. The data eliminate the hypothesis that a 2,3/2′,3′ tetramer is the catalytic unit of resolvase; this leads us to conclude that the 1,2 dimer is the form that not only binds DNA but also effects its breakage and reunion. In addition, the data suggest that the 1,2 dimers of resolvase bound to the two copies of site I in the synaptic complex do not communicate with the rest of the complex by way of the 2-3′ interface. This is a somewhat weaker inference, since in the complementation experiment, sites II and III are presumably occu-

pied by resolvase mutants (S10L) that have intact 2-3′ interfaces (including residue Arg^2); thus, the Arg^2 connection (which is made twice at each interface) would only be partially eliminated.

The proposal that the catalytic unit of resolvase consists of a pair of 1,2 dimers has two implications. First, the 1,2 dimer, when bound to site I, must be appreciably distorted from its crystallographic conformation to allow access of the two Ser^{10} residues to the crossover point. We have recently presented other data to support this view (*16*), and Rice and Steitz (*17*) have proposed that a scissorlike movement at the 1,2 interface could bring the two active sites closer together. Second, the two site I–bound 1,2 dimers either have a 222-symmetrical way of interacting with one another that is as yet unrecognized (perhaps mediated by the COOH-terminal 63 residues of resolvase, which are not visible in any crystal forms of the intact protein), or are held by resolvase-resolvase interactions with the rest of the synaptic complex in an equivalent symmetrical manner (*17*) (these two alternatives are, of course, not mutually exclusive). Consideration of the DNA-invertases, a resolvase-related class of site-specific recombinases, exemplified by Hin and Gin suggests that a direct interaction between the two catalytic dimers must occur (*18*). The inversion systems have just one recombinase dimer binding site for each recombination partner, and although these systems use an accessory protein, Fis, pairing of recombination sites can be observed with Hin alone in the absence of Fis (*19*). In addition, Fis-independent mutants of Gin give recombination in the absence of the accessory protein, indicating that the recombinase dimer contains the information necessary for synapsis (*20*).

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21. Resolution reactions in 20 mM tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM NaCl were initially 10 μl and contained about 0.4 μg of supercoiled substrate DNA and the R172L mutant (1 U ≈ 0.1 μg) as indicated. After incubation for 5 min at 37°C, an additional 10 μl containing the S10L mutant (0.05 μg) or dilution buffer was added, and incubation was continued for a further 20 min. DNAs were then digested with the appropriate restriction enzyme (10 U, 60 min; Bst I for pNG345, Eco RI and Pst I for pNG210). Finally, proteinase K [2 μl of stock solution (10 mg/ml)] was added, and the mixture was incubated for 10 min.
22. DNase I footprinting of resolvase-DNA complexes was as described [N. D. F. Grindley *et al.*, *Cell* **30**, 19 (1982)]. The DNA fragment was a 250-bp Eco RI–Dde I fragment 3′–end labeled at the Eco RI site, proximal to site I^{G2T}.
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24. Resolution reactions were set up in two parts. First, a 10-μl reaction (*21*) containing the first mentioned resolvase mutant was incubated for 5 min at 37°C. An additional 10 μl of resolution buffer containing the second mentioned resolvase (or, if only one resolvase is listed, a second portion of the same mutant) was then added, and incubation was continued for a further 30 min before addition of Eco RI (5 U). The amount of each resolvase per 10-μl reaction volume was 0.5 U, to give a total of 1 U of resolvase (or resolvases) per 20-μl final volume.

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Calcium Sparks: Elementary Events Underlying Excitation-Contraction Coupling in Heart Muscle

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Spontaneous local increases in the concentration of intracellular calcium, called "calcium sparks," were detected in quiescent rat heart cells with a laser scanning confocal microscope and the fluorescent calcium indicator fluo-3. Estimates of calcium flux associated with the sparks suggest that calcium sparks result from spontaneous openings of single sarcoplasmic reticulum (SR) calcium-release channels, a finding supported by ryanodine-dependent changes of spark kinetics. At resting intracellular calcium concentrations, these SR calcium-release channels had a low rate of opening (~0.0001 per second). An increase in the calcium content of the SR, however, was associated with a fourfold increase in opening rate and resulted in some sparks triggering propagating waves of increased intracellular calcium concentration. The calcium spark is the consequence of elementary events underlying excitation-contraction coupling and provides an explanation for both spontaneous and triggered changes in the intracellular calcium concentration in the mammalian heart.

Excitation-contraction coupling in heart muscle is triggered by an increase in the concentration of intracellular calcium ($[Ca^{2+}]_i$) due to the sarcolemmal Ca^{2+} current (I_{Ca}), and this increase in $[Ca^{2+}]_i$ is amplified by the SR by a process known as Ca^{2+}-induced Ca^{2+} release (CICR) (*1–3*). CICR also exists in other types of muscle and in many cells with intracellular Ca^{2+} stores (*4, 5*). For the heart, studying CICR in situ has been difficult because of the complex microarchitecture of the muscle cell, which precludes direct examination of intracellular events at the level of single channels. Although the Ca^{2+}-release channel has been studied in reconstitution experiments in planar lipid bilayers and has been identified as the ryanodine receptor (RyR) (*6*), such experiments necessarily preclude examination of the regulation of the release channel in its intracellular environment. Using a laser scanning confocal microscope and the fluorescent Ca^{2+} indi-

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