Site-specific recombination in mammalian cells catalyzed by γδ resolvase mutants: implications for the topology of episomal DNA

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Abstract **We have transferred the prokaryotic γδ resolvase system to mammalian cells and present a comparative analysis of recombination by wild-type and two mutant resolvases (E124Q and E102Y/E124Q). Transient co-transfection assays using β-galactosidase as reporter for recombination reveal that episomal DNA does not contain a significant level of unconstrained negative supercoiling, since only mutant resolvases are recombination-proficient. We also show that the efficiency of recombination by the resolvase double mutant is comparable to that observed with Cre, which indicates that resolvase can be used as a new tool for controlled manipulations of episomal DNAs.**

Key words: Site-specific recombination; γδ resolvase; Mutant recombinase; Eukaryote; Episomal DNA; DNA topology

1. Introduction

The bacterial γδ (Tn*1000*) transposon-encoded resolvase belongs to the resolvase/invertase sub-family of conservative site-specific DNA recombinases. Its natural function is to resolve, through recombination, the co-integrate structure that is generated during transposition [1]. Resolvase recognizes a 114 bp DNA segment called res, which is composed of three binding sites (I–III) for resolvase dimers. Two res must be present as direct repeats on the same negatively (−) supercoiled DNA molecule. Only this orientation of sites allows for the formation of a functional synaptic complex, the so-called synaptosome, which entraps three (−) supercoils [2]. Intramolecular recombination catalyzed within the synaptosome thus results in excision of the DNA segment between res sites.

An important prerequisite for efficient recombination by wild-type resolvase in *Escherichia coli* and in vitro is the presence of unconstrained (−) supercoiling in the substrate. Supercoiling is required for the assembly of a functional synaptosome and, in addition, energetically drives the DNA strand exchange reaction once the DNA has been cleaved at sub-binding sites I of the paired res [3]. We have transferred the resolvase system to mammalian cells and employ in this study wild-type and two γδ resolvase mutants, E124Q and double mutant E102Y/E124Q. Both mutants are homologs of two previously characterized Tn*3* resolvase mutants and exhibit the same phenotype, i.e. they efficiently perform recombination in vitro on topologically relaxed DNA substrates containing two copies of res [4]. The double mutant, in addition, is proficient at catalyzing recombination on substrates containing two isolated I sites of res as direct or inverted repeats. However, the efficiency of the latter reaction is still significantly enhanced by (−) supercoiling of the substrate [4]. A comparative study of recombination by wild-type and mutant resolvase inside mammalian cells should therefore help to further elucidate the topological state of episomal and, eventually, genomic DNA.

2. Materials and methods

2.1. Expression and substrate vectors

Expression vectors for wild-type and mutant resolvases are derivatives of pPGKCre (K. Fellenberg, University of Cologne). The resolvase genes were cloned by PCR using primers (P-γδA) 5′-GATACTGCAGCATGCGACTTTTTGGTTACGCACGGGTATCA-3′, (P-γδB) 5′-GATATCTAGATTAGTTGCTTTCATTTATTACTTTATA-3′, (P-γδ124Q) 5′-CGACAGAGAATACTACAGCGTACCAATGAA-3′, (P-γδ124Qanti) 5′-TTCATTGGTACGCTGTAGTATTCTCTGTCG-3′, (P-γδ102Y) 5′-AGTACCGATGGGTATATGGGTAAAATGGTT-3′, (P-γδ102Yanti) 5′-AACCATTTTACCCATATACCCATCGGTACT-3′, and (P-2972) 5′-CATATCTAGACTATTAAACCTTCCTCTTCTTCTTAGGGTTGCTTTCATTTATTACTTTATA-3′. Wild-type resolvase was generated with primers P-γδA and P-γδB, and DNA isolated from *E. coli* strain DH5α, which contains the γδ transposon on the F′ plasmid, served as template. The nuclear localization signal (NLS) variant (pPGKγδNLS) was generated using P-2972 instead of P-γδB. The resulting PCR fragments replaced the Cre gene in pPGKCre, using *Xba*I and *Pst*I. pPGKγδ124 was generated by assembly PCR using P-γδA and P-γδ124Qanti, as well as P-γδB and P-γδ124Q as primer pairs. pPGKγδ served as template. pPGKγδ102 was also generated by assembly PCR using P-γδA and P-γδ102Y, as well as P-γδB and P-γδ102Yanti as primer pairs. pPGKγδ124 served as template. The corresponding NLS-carrying variants were generated as described for pPGKγδNLS.

Substrate vectors are derivatives of pCH110 (Pharmacia). First, the prokaryotic promoter driving the *lacZ* gene in pCH110 was deleted by PCR and the *lacZ* gene was re-inserted. The neomycin gene was isolated from pSV2Neo [5] using *Sma*I and *Bgl*II, and subsequently flanked by two res sites which were generated by PCR employing pZWX1 [6] as template. After ligation, the res-neo-res cassette was cleaved with *Hin*dIII and inserted between the SV40 promoter and the *lacZ* gene of the modified pCH110 vector. The promoter proximal to res was modified at two residues in order to eliminate two ATGs, thus generating pCH-RNRZ. pCH-SNSZ and pCH-RLNRLZ are derivatives of pCH-RNRZ, and were generated by PCR. The corresponding recombined product vectors of pCH-RNRZ and pCH-RLNRLZ were generated through transformation into DH5α or 294-Cre [7], respectively. pCH-SZ was generated by PCR.

Plasmid DNAs were isolated from *E. coli* strains JC5547, BMH8117, DH5α or 294-Cre using affinity chromatography (Qiagen, Germany). Expression and substrate vectors were sequenced using the fluorescence-based 373A system (Applied Biosystems). PCRs were usually performed with 30 cycles using the 'Master MixQ Kit' (Qiagen, Germany). The reaction temperatures were calculated based on

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Abbreviations: res, γδ recombination site; lox, Cre recombination site; β-gal, β-galactosidase activity

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the respective primer sequences and the length of the expected product.

2.2. Cell culture and in vivo recombination assays

Transient resolvase expression and recombination assays employed CHO cells cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, streptomycin (0.1 mg/ml), and penicillin (100 U/ml). Transient co-transfection assays were performed with supercoiled expression and substrate vectors at a molar ratio of about 3:1 and lipofection (FuGene™6; Boehringer Mannheim, Germany). About 2×10^5 cells were transfected with 2 μg of mixed plasmid DNA. Transfection efficiencies were usually in the range of 20–30%. Cells were analyzed 72 h post transfection by either cell staining or determining the β-galactosidase activity (β-gal).

2.3. β-Gal assays

Cells were stained after fixation with 0.5% (w/v) glutaraldehyde through incubation with a solution made up of 5 mM $K_3Fe(Cn)_6{\cdot}3H_2O$, 2 mM $MgCl_2$, and 4 mg/ml X-Gal in phosphate-buffered saline. The number of stained cells was determined microscopically after 7–8 h of incubation at 37°C. β-Gal activity was determined in cell lysates using the Galacto Light Kit (Tropix, Perkin Elmer), as recommended by the manufacturer. Relative light units (RLU) were determined in a Lumat LB9501 luminometer (Berthold), and normalized to the amount of protein (μg) in each lysate determined by the Bio-Rad protein assay.

2.4. Immunoblotting

Cell lysates from transiently transfected cells were prepared by boiling the harvested cells in sample buffer for 5 min. Proteins were separated through 12.5% SDS polyacrylamide gels and transferred onto a nitrocellulose membrane (Immobilon P, Millipore). The membrane was blocked with 1% blocking solution and incubated with mouse polyclonal antibodies raised against wild-type resolvase at a 1:3000 dilution. Peroxidase-conjugated secondary antibodies were used for detection (Boehringer Mannheim, Germany). Purified resolvase was used as a control [6].

3. Results

3.1. Expression of wild-type and mutant resolvases in CHO cells

We constructed eukaryotic expression vectors which contain the coding region for wild-type or mutant resolvase under the control of the phosphoglycerate kinase promoter (PGK). Two sets of vectors were generated: one containing an NLS and a corresponding set (Fig. 1A) lacking one fused to the C-terminal end of resolvase genes. At 72 h post transfection into CHO cells, we could detect resolvase by Western blotting using mouse polyclonal antibodies raised against wild-type resolvase (Fig. 2, lanes 3–8). The parental expression vector (pPGKCre) containing the Cre gene (lane 1) and purified resolvase (lane 2) served as controls. The results also show that the presence of the NLS has a profound effect on the amount of resolvase detectable in cell lysates (lanes 4, 6, and 8). However, the amount of each resolvase variant generated from the same set of expression vectors seems to be comparable. Identical results were obtained after transfection into HeLa and NIH3T3 cells (data not shown). We conclude that presumably full-length resolvase protein is produced by different cell lines.

3.2. Only mutant resolvases are recombination-proficient

In order to test whether wild-type and/or mutant resolvase can perform recombination in CHO cells, we constructed a substrate plasmid termed pCH-RNRZ (Fig. 1B). This vector contains two res as direct repeats flanking the neomycin resistance gene (*neo*). This recombination cassette, in turn, is flanked by the SV40 promoter (SV40) and the *lacZ* gene. Hence, recombination between res should lead to deletion of the *neo* gene and the subsequent expression of β-gal.

We introduced pCH-RNRZ together with each of the six expression vectors for resolvase into CHO cells. Cell extracts were prepared 72 h after transfection and recombination was monitored by determining the normalized β-gal activity. As a control to determine the highest achievable level, set at 100%, we co-transfected the recombined product vector pCH-RZ with pPGKCre. The results (Fig. 3A) show that cells expressing mutant resolvase, either with or without NLS, exhibit about 60% β-gal activity. This level is even higher (about 80%) when compared to a second control, i.e. pCH-RZ co-transfected with the expression vector for the double mutant, pPGKγδ102. In contrast, cells co-transfected with pPGKγδ or pPGKγδNLS and pCH-RNRZ exhibit only negligible β-gal activity (5%). This low level is comparable to two other controls: the substrate pCH-RNRZ co-transfected with pPGKCre, and pPGKγδ102 transfected alone.

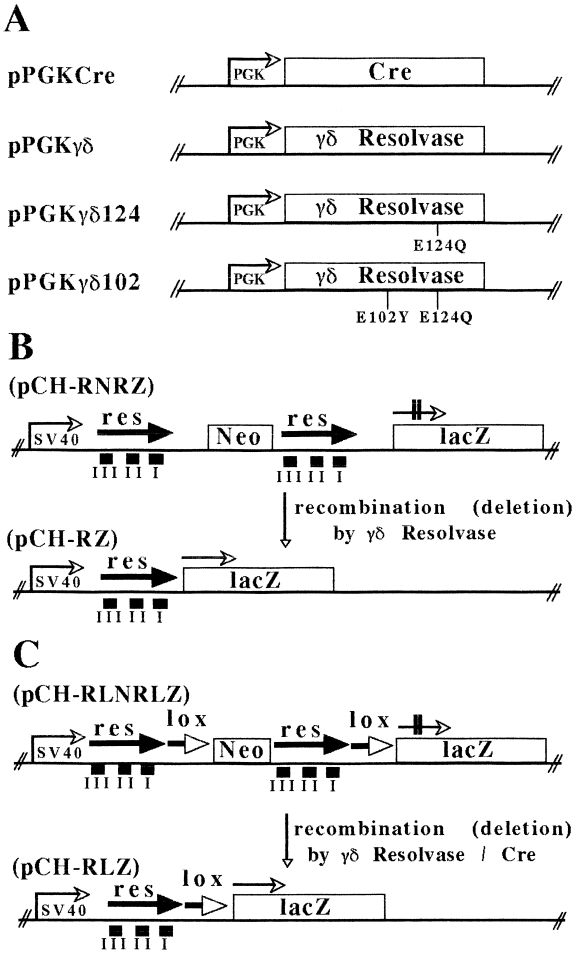

Fig. 1. Resolvase expression and substrate vectors. A: Relevant genetic elements present on expression vectors for Cre, wild-type or two mutant resolvases are schematized. A corresponding second set of vectors for resolvase, labelled with the postscript NLS, carries a SV40 large tumor antigen NLS fused to the C-terminal end of resolvase (not shown). B: Standard substrate for resolvase. C: Standard substrate for both resolvase and Cre. The orientation of recombination sites is indicated by arrows. The positions of resolvase subbinding sites within res are demarcated by black rectangles and labelled I–III. See text for details.

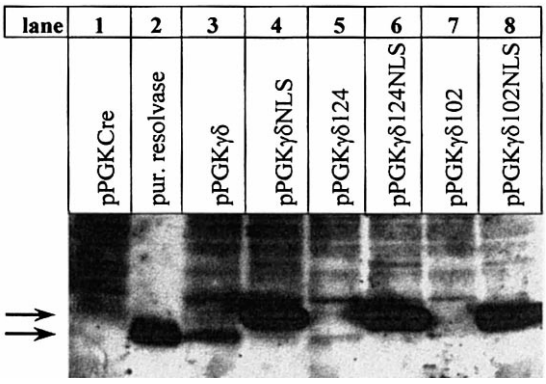

Fig. 2. Western analysis. Wild-type and mutant resolvases expressed in CHO cells. The higher and lower arrows point to the position of resolvase with and without an NLS, respectively.

We also determined the fraction of X-gal-stained cells 72 h after co-transfection of substrate and expression vectors. The result of a representative experiment (Table 1) reveals that both mutants generate a significant fraction of stained cells if compared again to the control employing the recombined substrate (pCH-RZ), set at 100%. However, this fraction is rather low and comparable to a negative control (pPGKCre) when either pPGKγδ or pPGKγδNLS was used. We conclude that within the limits of detection by these methods both mutant resolvases, but not the wild-type, catalyze recombination on episomal substrates in mammalian cells.

3.3. Recombination on isolated I sites of res

We were interested to test whether mutant resolvases can recombine two copies of isolated I sites of res. We therefore constructed pCH-SNSZ, which is identical to pCH-RNRZ except that sub-sites II and III of each res are deleted (compare Fig. 1B). Recombination assays employing the corresponding product vector pCH-SZ and pPGKCre as control (100%) were performed as described above. The results show (Fig. 3B) that only the resolvase double mutant recombines this substrate in CHO cells. However, the efficiency of this reaction is significantly reduced compared to recombination on pCH-RNRZ.

3.4. Cre and the resolvase double mutant exhibit comparable activities

In order to compare the recombination efficiency of the resolvase double mutant with that of Cre, we generated pCH-RLNRLZ. This substrate is identical to pCH-RNRZ, but contains in addition two recombination sites (lox) for Cre as direct repeats also flanking the *neo* gene (Fig. 1C). Hence, recombination by either resolvase or Cre deletes the *neo* gene and results in the subsequent expression of the *lacZ* gene. Co-transfections were performed with pPGKγδ102NLS and pPGKCre as described above. The corresponding recombined product vector was used in each case as control (100%).

Table 1
Recombination analyzed by cell staining

Co-transfected plasmids	Stained cells (%)
None	0
pCH-RZ+pPGKγδ102NLS	100
pCH-RNRZ+pPGKCre	9
pCH-RNRZ+pPGKγδ	4
pCH-RNRZ+pPGKγδNLS	11
pCH-RNRZ+pPGKγδ124NLS	44
pCH-RNRZ+pPGKγδ102NLS	96

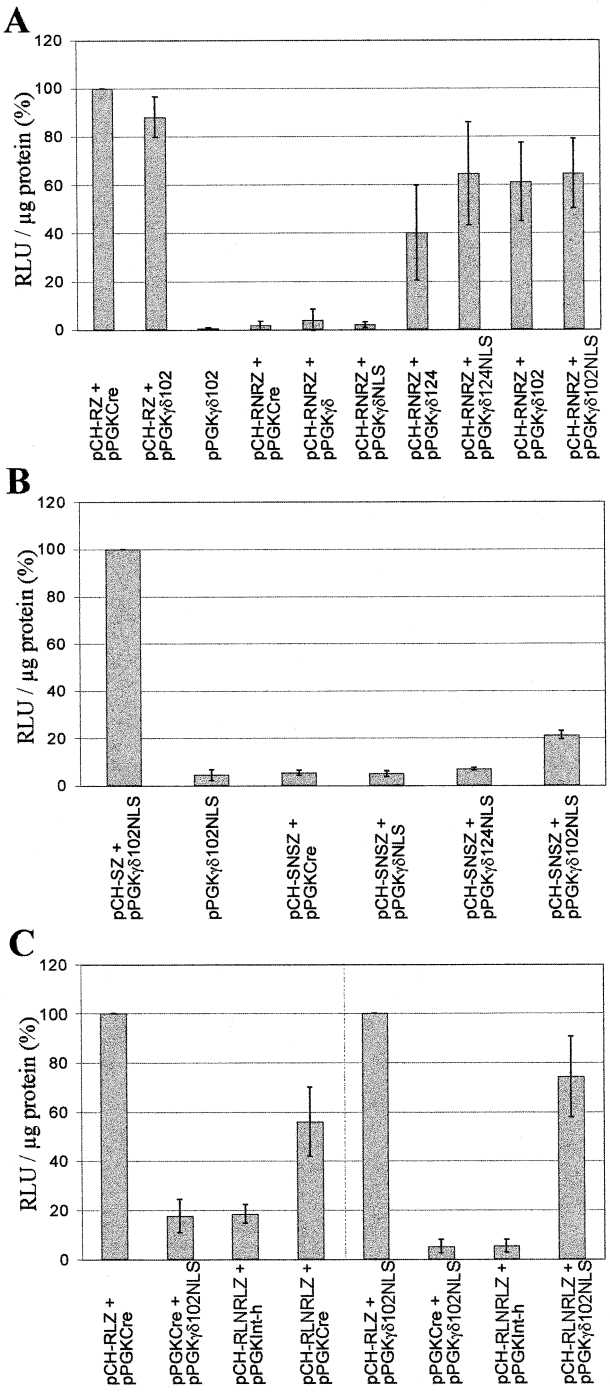

Fig. 3. Analysis of recombination by β-gal assays. A: Recombination on pCH-RNRZ containing two complete res. B: Recombination on pCH-SNSZ containing two isolated copies of I sites of res. C: Recombination on pCH-RLNRLZ containing res and lox sites. As an additional control, in C we used an expression vector for a phage λ integrase mutant, Int-h, co-transfected with pCH-RLNRLZ [11]. In each case, data were collected from four to six separate transfections assays each employing between two and four wells containing about 2×10^5 CHO cells. The graphs show the mean values with standard deviations indicated by vertical lines. See text for details.

The results show that the double mutant is at least as efficient as Cre at performing recombination on episomal substrates (Fig. 3C).

4. Discussion

We have demonstrated that two mutant γδ resolvases can catalyze intramolecular recombination on episomal res inside mammalian cells. Recombination by wild-type resolvase, however, is not detectable. Based on the fact that only mutant resolvases recombine topologically relaxed substrates [4], a major conclusion is that episomal DNA does not contain a significant level of unconstrained (−) supercoiling over at least the time course of the experiment. This interpretation is supported by the result that only the double mutant recombines a substrate containing isolated I sites of res, albeit with a significantly reduced efficiency compared to recombination on a standard substrate.

Since we used (−) supercoiled plasmids for co-transfection, a second conclusion is that plasmid supercoiling must be relaxed once the DNA is introduced to the cells. This could be due to the action of topoisomerases [8] and/or the formation of nucleosomes on the internalized plasmids, a process which would consume unconstrained (−) supercoils. However, based on a topological analysis of plasmid DNA isolated from CHO cells 72 h post transfection, we found no evidence for nucleosome formation, i.e. the plasmid DNA was topologically relaxed (data not shown). Future comparative studies employing wild-type and mutant resolvase in reporter cell lines should also help to elucidate the topological state of genomic target sites and how it might be affected by, for example, transcription and the influence of topoisomerase or histone acetylase inhibitors [8,9].

A third conclusion from our study is that the resolvase double mutant performs recombination on episomal substrates with an efficiency comparable to that of Cre. The latter recombinase is used widely in studies elucidating the relevance and function of particular genes of interest in animal model systems [10]. If recombination by the resolvase double mutant is also efficient on genomic target sites, the available possibilities for controlled in vivo and ex vivo manipulations of eukaryotic genomes will be expanded.

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