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Daxx Interacts With Phage ΦBT1 Integrase and Inhibits Its Recombination*

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Abstract The bacterial phage ΦBT1 integrase is a promising tool due to its site-specific transgene character. It enriches the site-specific transgenic tools and provides the possibility for multiple site-specific transgenic manipulations. To improve its safety as a vector of gene therapy, it is necessary to investigate the potential interactions between ΦBT1 and proteins in mammalian host cells. Yeast mating and co-immunoprecipitation assay indicated that a tetrapeptide 433RFAL436 in ΦBT1 integrase was responsible for ΦBT1 and Daxx interaction. It was also demonstrated that over-expression of Daxx could reduce ΦBT1 mediated recombination rate in 293T cells by using ΦBT1 report system. It is the first time to identify a cellular protein interacting with ΦBT1 integrase and inhibiting its recombination efficiency. This result might be useful for improving the ΦBT1 integrase mediated transgene methods and directing the selection of target cells for ΦBT1 integrase.

Key words ΦBT1 integrase, interacting proteins, Daxx
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The bacterial phage ΦBT1 integrase belongs to a large serine recombinase family, members of which have a serine residue at their catalytic sites[1]. There are at least 30 members in this family and some of them have been studied, for example, ΦC31, Bxb1, ΦRv1 and TnpX [2-5]. It could efficiently catalyze the recombination reaction between the attachment sites in the phage (attP) and those in bacterial (attB) genomes, resulting in phage DNA integration into the bacterial host genome without the need for cofactors. The integration is unidirectional, and this ability has allowed the phage integrase to become a very useful tool for a multitude of applications in bacterial and mammalian genetics. The mechanisms of DNA cleavage and synapsis formation during ΦBT1 mediated recombination have been studied and the minimal sizes of the attB and attP were determined to be 36 and 48 bp, respectively[6-7]. A limited number of pseudo-attachment (pseudo-attP) sites have been identified in mammalian genomes and ΦBT1 integrase system has been used to correct phenylketonuria in mice[8-11].

In order to safely and efficiently use ΦBT1 integrase as a tool for gene therapy, potential interactions between ΦBT1 integrase and proteins in mammalian host cells should be studied. The organization of the ΦBT1 genome is highly similar to that of ΦC31, and the majority of gene products are closely related[12]. In our previous study, we found that a tetrapeptide 451RFGK454 in the C-terminus of a phage ΦC31 integrase was necessary for ΦC31 interaction with Daxx[13]. Interestingly, here we found a similar tetrapeptide 433RFAL436 in C-terminus of ΦBT1 integrase, and it was proved also a Daxx-binding motif. We further demonstrate that the interaction between Daxx and ΦBT1 integrase can influence the activity of ΦBT1 integrase.

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1 Materials and methods

1.1 Cell culture and transfections

293T cells were purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum at 37℃ in a 5% CO_2 humidified atmosphere. Plasmid DNAs were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

1.2 Plasmid construction

To construct a pLexA-ΦBT1 plasmid, the open reading frame (ORF) of ΦBT1 integrase was amplified from pFDZ15 (kindly provided by Prof. Xiaoming Ding) with a pair of primers: 5′ GAGGATCCTGTC-GCCCTTCATCGCTC 3′ and 5′ CCGCTCGAGCTA-CAGCGCCGCAAGCTC 3′. The PCR product was inserted into the plasmid pLexA (Clontech) between the *Bam*H Ⅰ and *Xho* Ⅰ sites. Plasmids that express mutant ΦBT1, namely, pLexA-ΦBT1 (Del433-436), pLexA-ΦBT1 (L436N) and were generated by a PCR-based point specific mutation strategy with the primers shown 5′ GCCGCCGCTGCGGAGCAGCA-CGACCTAGCGGGGGTG 3′, 5′ CACCCCCGCTA-GGTCGTGCTGCTCCGCAGCGGCGGC 3′, 5′ GC-GGAGCGCTTCGCCAATCAGCACGACCTAGCG 3′, and 5′ CGCTAGGTCGTGCTGATTGGCGAAGCG-CTCCGC 3′. The above obtained ΦBT1 ORF was inserted into pCMV-Myc(Clontech) between *Sfi* Ⅰ and *Not* Ⅰ sites to produce Myc-ΦBT1. It was also inserted into pEGFP-C3(Clontech) between *Xho* Ⅰ and *Bam*H Ⅰ sites in the frame with the C-terminus of EGFP to generate pEGFP-ΦBT1 that expresses EGFP-ΦBT1 fusion protein.

The ORF of human Daxx was obtained from a human fetal brain cDNA library (Clontech) by amplification with primers 5′ CGAATTCTTATGGC-CACCGCTAACAGCATC 3′ and 5′ CGCCTCGAG-CTAATCAGAGTCTGAGAGCAC 3′. The cDNA was inserted into pCMV-HA (Clontech) between *Eco*R Ⅰ and *Xho* Ⅰ to generate HA-Daxx. The plasmids pDsRed-Daxx and pB42AD-Daxx were constructed as described previously[13].

The plasmids mentioned above were all verified by sequencing analysis.

1.3 Yeast-mating assay

The MATCHMARKER LexA two-hybrid system was purchased from Clontech. The interactions between ΦBT1 integrase and Daxx and their derivatives were detected using pB42AD-Daxx transferred EGY48 [p8opLacZ] and pLexA-ΦBT1/ pLexA-ΦBT1 (Del433-436)/pLexA-ΦBT1 (L436N) transferred YM4271. The assay was performed following the standard mating test protocol provided by the manufacturer.

1.4 Intracellular localization of ΦBT1 integrase and Daxx

Plasmids expressing the fusion proteins, pEGFP-ΦBT1 and pDsRed-Daxx were co-transfected into 293T cells. 24 h later, cells were fixed in 4% paraformaldehyde and visualized using an Olympus LX70 microscope. The nuclei were stained with DAPI (4′, 6 -diamidino-2-phenylindole) as an internal nuclei location marker.

1.5 Co-immunoprecipitation and Western blotting

For immunoprecipitation, cells were lysed with lysis buffer (20 mmol/L Tris pH 7.5, 100 mmol/L NaCl, 0.5% NP-40, 0.5 mmol/L EDTA, 0.5 mmol/L PMSF, 0.5% protease inhibitor cocktail (Roche)). The lysates were then immunoprecipitated with the indicated antibodies for 2 h at 4℃. 20 μl pre-washed protein A/G Sepharose beads (Santa Cruz) were added to each extract and shaken overnight. The beads were washed three times with lysis buffer and boiled in 2 × loading buffer. Protein samples were then separated on SDS-PAGE and transferred to a nitrocellulose membrane, which was blocked in 5% skim milk in TBST and probed with the indicated antibodies. The following antibodies were used: anti-Myc mouse monoclonal antibodies and rabbit polyclonal anti-Daxx antibodies were purchased from Santa Cruz Biotechnology; Secondary antibodies, HRP-conjugated anti-rabbit IgG or anti-mouse IgG were obtained from Sigma.

1.6 ΦBT1 integrase activity assay

ΦBT1 integrase mediated recombination in mammalian cells was measured by using a ΦBT1 recombination report plasmid, pBTPL, which contains a structure of pCMV-attB-BGH polyA-attP-Luciferase. Upon ΦBT1 expression, it will mediate recombination between attB and attP sites and remove BGH polyA from the inserted structure, resulting in expression of luciferase(Figure 1). For the integration activity assay, 293T cells were plated in 12-well plates and transfected with 1 μg of Myc-ΦBT1 together with 200 ng of pBTPL per well. To normalize the transfection

efficiency, 2 ng of pRL plasmid that expressed renilla luciferase was co-transfected per well. At 24 h post transfection, cell extracts were prepared and assayed for luciferase activity according to the manufacturer's protocols (Promega).

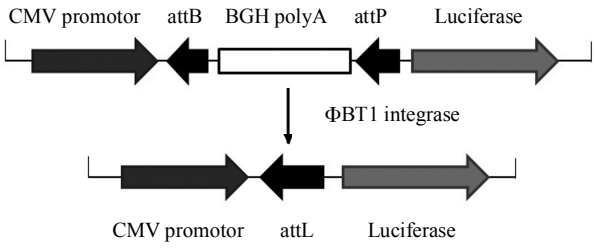

Fig. 1 Schematic illustration of the reporter system

Upon ΦBT1 expression, the integrase could mediate recombination between attB and attP sites and remove BGH polyA, resulting in expression of luciferase.

2 Results

2.1 Daxx interacts with ΦBT1 integrase

Firstly, we carried out yeast-mating assay to detect whether ΦBT1 integrase could bind to Daxx. The results showed that there was a robust binding between the two proteins (Figure 2). For further characterizing the binding site of ΦBT1 integrase, we generated two mutant ΦBT1 constructs, one with a deletion of the tetramer 433 ～436 residues (pLexA-ΦBT1 (Del433-436)) and the other was a point mutation construct (pLexA-ΦBT1 (L436N)). The yeast-two-hybrid assay showed no interaction between (pLexA-ΦBT1 (Del433-436)) and Daxx. Moreover, mutations at the Leu436 residue of ΦBT1 (pLexA-ΦBT1 (L436N)) also alter its binding to Daxx, suggesting that it played an important role in ΦBT1 and Daxx interaction.

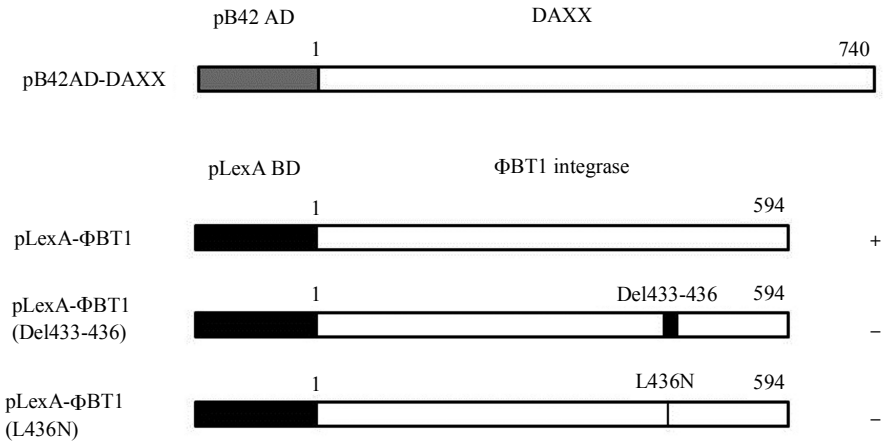

Fig. 2 Yeast two-hybrid assay of Daxx and full-length or mutants of ΦBT1 integrase

"+" indicates interaction between the two proteins, while "–" indicates no interaction.

The interactions between ΦBT1 integrase and Daxx were further verified by co-immunoprecipitation analysis. 293T cells were transfected with either Myc-ΦBT1 or a control plasmid pCMV-Myc. Endogenous Daxx was able to precipitate Myc-ΦBT1 but no non-specific interaction was seen in empty vector transfected cells, see Figure 3a, 3b. Taken together, the results of the yeast mating assays and co-immunoprecipitation indicated that ΦBT1 binds specifically with Daxx *in vivo*.

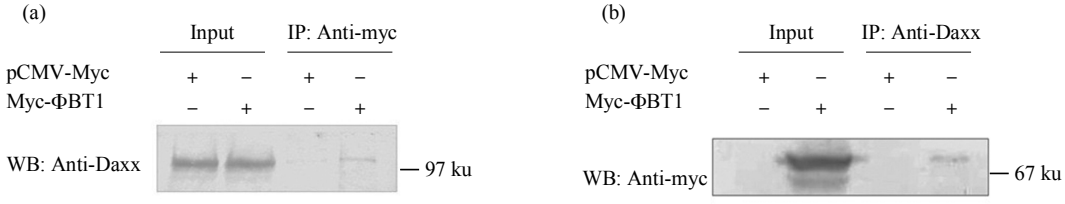

Fig. 3 Interaction between endogenous Daxx and ΦBT1 integrase

(a) The mouse anti-Myc antibody successfully precipitated endogenous Daxx in cells transfected with Myc-ΦBT1, and no non-specific interaction was seen in empty vector transfected cells. (b) Rabbit anti-Daxx antibody could also precipitated Myc tagged ΦBT1.

2.2 Subcellular co-localization of ΦBT1 integrase with Daxx

To demonstrate the intracellular localization of Daxx and ΦBT1 integrase, plasmids of pDsRed-Daxx and pEGFP-ΦBT1 were co-transfected into 293T cells. As shown in Figure 4, the red fluorescent Daxx fusion protein was mainly found as spots within the nucleus, while the ΦBT1 integrase was mainly dispersed in the cytoplasm, low levels of expression were detected in the nucleus of some cells.

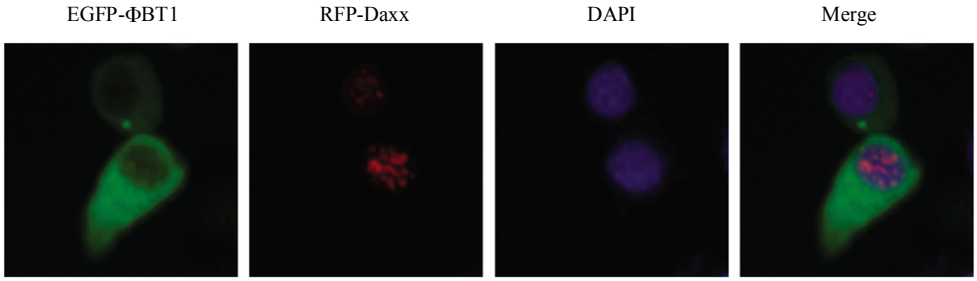

Fig. 4 Subcellular co-localization of Daxx and ΦBT1 integrase

Human 293T cells were co-transfected with pDsRed-Daxx and pEGFP-ΦBT1, and the subcellular location of the two proteins was analyzed.

2.3 Daxx inhibits ΦBT1 integrase-mediated recombination

To determine whether the interaction between Daxx and ΦBT1 integrase could affect the recombination efficiency, we constructed a ΦBT1 integrase report system which contains a structure of pCMV-attB-BGH polyA-attP-Luciferase. Upon ΦBT1 expression, the integrase could mediate recombination between attB and attP sites and remove BGH polyA, resulting expression of luciferase. The efficiency of recombination was expressed as relative luciferase activity folds of the cultures transfected without ΦBT1. As shown in Figure 5, in control group, when cells were transfected with ΦBT1 integrase expression plasmid, Myc-ΦBT1, the relative luciferase activity increased as (4.39±0.47) folds of the cells transfected with empty vector, indicating that the report system was functional in 293T cells. However, the recombination rate declined significantly when Daxx was co-expressed with ΦBT1 integrase, to (2.13±0.10) folds ($P < 0.05$). Thus, we suggested that Daxx can inhibit the integration efficiency of ΦBT1 integrase.

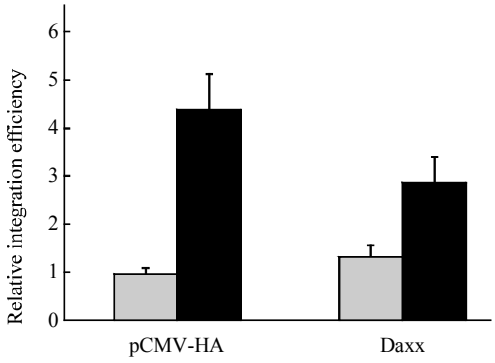

Fig. 5 Effects of Daxx over-expression on efficiency of ΦBT1 integrase mediated recombination in 293T cells

Cells were transfected with pRL and pBTPL together with indicated plasmids respectively. The efficiency of recombination was expressed as relative luciferase activity folds of the cultures transfected without ΦBT1. □: pCMV-Myc; ■: ΦBT1 integrase.

3 Discussion

In the present study, we discovered that the residues 433RFAL436 in C-terminus of ΦBT1 integrase interacted with cellular Daxx, and increasing expression of Daxx could result in reducing of recombination efficiency of ΦBT1 integrase. Though strong evidences provided by the yeast mating and co-immunoprecipitation assays supported a direct interaction between ΦBT1 integrase and Daxx, these two proteins co-localized poorly in cells. To sovle this question, we conducted an immunofluorescence assay to ascertain the localization of ΦBT1 integrase, and the result was consistent with those using fusion proteins. ΦBT1 integrase was still dispersed in the cytoplasm, (Figure S1 , Supplementary online, http://www.pibb.ac.cn/cn/ch/common/view_abstract.aspx?file_no=20110505&flag=1). ΦC31 integrase, the homolog protein of ΦBT1, has a similar localization and it also interacts with Daxx [13]. The two integrase

above mediated site-specific integrations of cellular genome only in nucleus, where they were poorly detected in fact for unknown reason. Thus, we arrive at the conclusion that there is an interaction between ΦBT1 integrase and Daxx in nucleus although it could not be detected in co-localization assay.

Site-specific recombinases have been widely used as tools for genome manipulation. Although ΦC31 and ΦBT1 belong to the same bacteriophage family, their corresponding attachment sequences are distinct. The pseudo-attP and pseudo-attB sites of the two integrases in the human genome also share no similarity [11, 14]. However, interestingly, both integrase could interact with Daxx and could be inhibited by it. It has been reported that Daxx could interact with several viral proteins and play an important role in the cellular intrinsic immune defense against the incoming viruses[15–17]. So we believe that Daxx may function as a cellular restriction factor in defending environment intruders and maintain the integrity and stabilization of genome though the precise biological process remains to be explored.

Some results demonstrated that the activity of the ΦC31 integrase is strongly cell type specific. Particularly in haematopoietic cells, only low activity was observed. It may partially because higher amounts of Daxx protein in haematopoietic cells and ΦC31 integrase recombination efficiency had been impaired [18]. In our study, we discovered Daxx could inhibit ΦBT1 integrase through similar approaches. These findings may give suggestion when implication of ΦBT1 integrase in genetic manipulating and gene therapy.

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Daxx 与 ΦBT1 相互作用并抑制其重组活性 *

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摘要　噬菌体整合酶 ΦBT1 因其具有可介导转基因位点特异性整合的能力而成为了基因治疗中一种有效的工具，它丰富了转基因载体的选择，并使得多位点特异性整合成为可能．为了能够更加安全有效地利用 ΦBT1 整合酶作为基因治疗载体，有必要了解 ΦBT1 整合酶与宿主细胞内蛋白质相互作用的情况．酵母配对实验与免疫共沉淀实验揭示了 ΦBT1 整合酶中 4 个氨基酸 433RFAL436 对整合酶与 PML-NBs 蛋白 Daxx 的结合起到了关键作用．通过进一步构建并利用 ΦBT1 整合酶哺乳动物细胞报告系统，证实过表达 Daxx 会抑制 ΦBT1 整合酶在 293T 细胞中的重组效率．以上结果表明，细胞内的蛋白质可以与 ΦBT1 整合酶发生相互作用并抑制其重组活性，对于改善 ΦBT1 整合酶介导的转基因操作以及选择 ΦBT1 整合酶靶细胞方面具有重要的参考意义．

关键词　ΦBT1 整合酶，互作蛋白，Daxx
学科分类号　Q7，Q34　　**DOI**: 10.3724/SP.J.1206.2011.00505

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附　　录

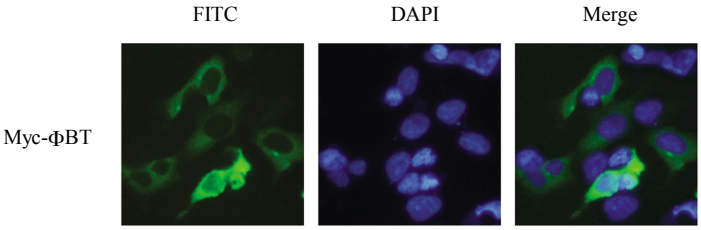

Fig. S1　The localization of ΦBT1 integrase in 293T cells

293T cells were plated on coverslips in 6-well plates and transfected with Myc-ΦBT1. 24 h later, cells were fixed and processed for immunofluorescence with anti-Myc antibodies. FITC-conjugated goat anti mouse antibody was used as secondary antibody. The nuclei were stained with DAPI.